Recent computational developments on CLIP-seq data analysis and microRNA targeting implications

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Abstract

Cross-Linking ImmunoPrecipitation associated to high-throughput sequencing (CLIP-seq) is a technique used to identify RNA directly bound to RNA-binding proteins across the entire transcriptome in cell or tissue samples. Recent technological and computational advances permit the analysis of many CLIP-seq samples simultaneously, allowing us to reveal the comprehensive network of RNA–protein interaction and to integrate it to other genome-wide analyses. Therefore, the design and quality management of the CLIP-seq analyses are of critical importance to extract clean and biological meaningful information from CLIP-seq experiments. The application of CLIP-seq technique to Argonaute 2 (Ago2) protein, the main component of the microRNA (miRNA)-induced silencing complex, reveals the direct binding sites of miRNAs, thus providing insightful information about the role played by miRNAs(s). In this review, we summarize and discuss the most recent computational methods for CLIP-seq analysis, and discuss their impact on Ago2/miRNA-binding site identification and prediction with a regard toward human pathologies.

Key words: RNA immunoprecipitation; bioinformatics workflow; computational guideline; large-scale analysis; quality management; microRNAs; human pathologies

Introduction

RNA-binding proteins (RBPs) bind RNAs to regulate their fate, function, localization or secondary structure [1] to ultimately modulate many biological processes including cell apoptosis, growth, fate and differentiation [2–4]. RBPs possess modular structure composed by at least one domain to directly bind either single- or double-stranded RNA, such as the RNA recognition motif, the zinc-finger domain, the KH domain and the double-stranded RNA-binding domain [5]. RNA-binding domains recognize primarily the RNA sequence, the RNA shape or both [6]. Because of the high versatility of the RNA-binding domains and the presence of some of them in each RBP [7], the full understanding of the RBP mode of binding is a challenging quest. To comprehensively uncover the RNA-protein interactions network in a genome-wide manner, Cross-Linking ImmunoPrecipitation associated to high-throughput sequencing (CLIP-seq) was...
recently developed [8]. Nowadays, CLIP-seq analysis has become one of the mainstream methods to study RNA metabolism and has led to important discoveries in different fields of molecular and cellular biology [9–14]. Of particular interest for the community of RNA biologists and beyond is the application of CLIP-seq methods to Argonaute 2 (Ago2) protein, which together with microRNAs (miRNAs) form the miRNA-induced silencing complex (miRISC) [15]. miRISC targets mRNAs through partial sequence complementarity between the miRNA and the target sequence, promoting degradation or translation inhibition of the target mRNA [16]. miRNAs regulate many biological processes, including cell proliferation, differentiation and death in both physiological and pathological events [16]. Because CLIP-seq analysis of Ago2 is meant to identify the precise binding sites of miRNAs [9, 12, 17] but not of the protein itself, the downstream and validation steps of the analysis are different from those performed for other RBPs: binding features of miRNAs follow different rules to that of RBPs. On the other hand, if we consider each miRNA loaded into Ago2 as a different RBP [18], Ago2 CLIP-seq may be taken as universal example of the mode of binding to cognate RNAs.

CLIP-seq protocol has many steps involving sample preparation, sequencing and bioinformatics analysis. Briefly, RNA of ultraviolet cross-linked cells or tissue lysates is partially digested by enzymatic reaction into small fragments of about 50–100 nucleotides (nts), the RBP of interest is immunoprecipitated and the RBP-RNA complex is recovered by acid phenol/chloroform extraction, RNA adapters are ligated, RNA template is reverse transcribed and finally high-throughput sequenced. Several protocol variants for sample preparation have been proposed, which mainly include the most popular High-Throughput Sequencing of RNA isolated by CLIP (HTS-CLIP) [9, 10], the Photonucleoside-Enhanced-CLIP (PAR-CLIP) [11–13] and the individual-nucleotide resolution CLIP (iCLIP) [19]. Detailed differences among these three variants and other more recent protocols have been previously discussed by others [20, 21]. An additional variant of the CLIP-seq, named CLASH, has been developed by Helwak et al. [22], and it is particularly appropriate to Ago2 CLIP-seq. Briefly, CLASH allows high-throughput mapping of small RNA:RNA-binding interaction by adding an intramolecular RNA ligation step during the sample preparation. CLASH approach and analysis have been previously reviewed by Broughton and Pasquinelli [23].

After high-throughput sequencing, the bioinformatics analysis workflow starts by the preprocessing to filter out the low quality and duplicate reads, and to map them onto the genome or the transcriptome of reference. Afterward, to assess real signal over the noise background, the reads are processed by peak-calling programs. Called peaks are further analyzed for functional, structural and biochemical characterizations of the RNA–protein interaction, including motif discovery, expression profile and gene ontology.

Importantly, because recent advances in sequencing technologies and bioinformatics analyses enable us to handle many CLIP-seq samples simultaneously, it is important to optimize a bioinformatic pipeline that can facilitate the work of researchers in obtaining unbiased and high-quality data. Despite great efforts from researchers to streamline CLIP-seq analysis, much remains to be improved on both experimental (e.g., the quality of the antibody used for the immunoprecipitation) and computational procedures. In fact, because of the complexity of the large data set coming from high-throughput technologies such as CLIP-seq, a correct interpretation of the data is facilitated by a proper and an accurate data analysis with refined and optimized computational tools. In this review, we describe the computational protocol for CLIP-seq analysis, discuss the latest bioinformatics developments for data processing and mining and provide advices for data analysis interpretation. We discuss the validity and the limitations of emerging programs for each step of the CLIP-seq analysis and the quality measurements currently available for specific tasks, by providing concrete examples on a case study: an in-house Ago2 HITS-CLIP data set generated from stem cells [24] (GEO accession: GSE85219). For simplicity and limited space, we focus the scope of this review in providing a valuable computational guideline for the bioinformatics analysis of the three main variants of CLIP-seq analysis, namely, HITS-CLIP, PAR-CLIP and iCLIP. To help the readers, we provide in Supplementary Table S1 the Web links to download all the programs cited in the present review. Finally, we discuss how Ago2 CLIP-seq analyses have improved the miRNA-binding site prediction and the understanding of miRNA function in human pathologies.

Bioinformatics workflow for CLIP-seq analysis

In Figure 1, we have summarized the main computational steps for CLIP-seq analysis. Recently, few computational pipelines have been developed such as CIMS [25], CLIPSeqTools [26], CLIPZ [27] and PARCLIPsuite [11], which provide useful resources to deal with preprocessing steps and some of the main steps of the analysis, including peak-calling procedure. In the following sections, we describe step-by-step the bioinformatics workflow of the CLIP-seq analysis giving a quality overview of existing software and providing practical examples on an in-house data set for Ago2 HITS-CLIP experiments on P19 stem cells [24].

Preprocessing and read mapping onto the reference genome

The first step of the analysis is the preprocessing that involves adapter removal, filtering raw data according to read quality scores and collapsing reads with the exact sequence. While for the adapter removal, specific programs have been developed such as cutadapt [28] or Trimomatic [29], for the quality filtering, usually bioinformaticians develop ad hoc scripts. However, lately few programs have been developed, such as FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and PRINSEQ [30]. To quantify the differences of the strategies used by different preprocessing programs to filter reads based on quality scores and collapse duplicated reads, we applied FASTX-Toolkit, PRINSEQ and the CIMS pipelines to a published in-house Ago2 CLIP-seq data set from mouse P19 stem cells [24] (GEO accession: GSE85219). This analysis was run with the default tuning or the recommended parameters (see Supplemental Information). While some programs have tunable parameters, we forgo parameter optimization, which might have improved the results for some data sets, as this task may be beyond the ken of most users. The highest number of reads that survive the preprocessing step for the three replicates was obtained using PRINSEQ (Table 1). To inspect the quality of the reads after the preprocessing, we used the program FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) developed by S. Andrews. As shown in Supplementary Figure S1, although FASTX-Toolkit yielded reads with best quality score per sequence and per base,
this program filtered out much more reads compared with the other two programs (Table 1). These data indicate that FASTX-Toolkit is stringent to select high-quality reads. Altogether, PRINSEQ showed the best balance between quality and amount of reads, suggesting that this program uses a strategy that fits better for CLIP-seq data preprocessing than the other two programs. However, the balance of stringency and sensitivity can be tuned by changing the parameters of the programs to meet the needs of the researcher, such as the minimal quality of reads or their minimal length to be selected. Finally, the three methods achieved similar results regarding the collapse of duplicates, to remove redundant fractions of the data. A high redundancy of the data may be a consequence of low library complexity, which often occurs when samples are prepared from small amount of starting material or by performing too many polymerase chain reaction cycles for library construction.

Reads that survive the preprocessing steps are mapped onto the reference sequences that can be the complete genome, the transcriptome or sequences belonging to specific categories, such as 3’ untranslated regions (UTRs), long noncoding RNAs, small RNAs, etc. The most common algorithms used to perform this task are Novoalign (http://www.novocraft.com/products/novoalign/), STAR [31], Bowtie [32], RMAP [33], TopHat [34], Gsnap [35], SOAP [36] and BWA [37]. A few precautions have to be considered while setting the parameters of these programs to perform a gapped alignment onto the genome. In fact, these parameters permit to map reads that include deletions, mutations or insertions caused by enzymatic errors occurring during the sample preparation. Depending on the sample, it might be important to use adequate parameters to map reads on the exon junctions. In addition, an important issue concerns the consideration of multiple mapped reads (reads that map in
many loci). Although allowing for multiple read mapping would increase the number of usable reads and the sensitivity of peak detection, this may also cause an increase of false-positive peaks, as it was also suggested for ChIP-seq analysis [38].

To provide guidelines about the program to use, we ran two popular programs that achieved the best performances on RNA-seq data [39], namely, Novoalign and STAR, on the in-house Ago2 HITS-CLIP data set from P19 stem cells after preprocessing with the three aforementioned programs. For this analysis, we set the recommended parameters for Novoalign and used similar ones for STAR (Supplemental Information). As shown in Table 1, regardless the preprocessing program used, Novoalign mapped uniquely between 20 and 30% more reads than STAR. To test whether there is a qualitative difference on the genomic location of the reads mapped by Novoalign and STAR, we divided the genome in bins of 100 nucleotides and counted the number of bins in which reads map by the two programs. As shown in Supplementary Table S2, we found a relative small number of bins in common between the two programs. These data indicate that Novoalign is able to map reads in about 10% more of the genomic locations than STAR. Therefore, we concluded that differences in both quantitative and qualitative mapping performances exist between Novoalign and STAR. Giving that higher numbers of correctly and uniquely mapped reads would have a beneficial effect on the peak-calling step to discriminate real peaks over the background, we would recommend to use Novoalign. However, the full version of Novoalign is not freely downloadable, and the computational analysis can take much longer with the incomplete free version compared with STAR, which is freely downloadable. An alternative strategy is to map the reads with one program and afterward run the unmapped reads with a second program. Because this strategy was not benchmarked yet, it is unclear whether it is really advantageous. Finally, the mappability of multiple mapped reads can be dealt by tuning the minimal length of reads or using paired-end sequencing [38].

### Peak calling

Assessing peaks is a central step of the analysis to determine specific signal over the noise background for the identification of real binding sites. The number of identified peaks increases with the sequencing depth because weaker sites become statistically significant with a greater number of reads [40]. However, the optimal sequencing depth can only be experimentally evaluated, as it depends on the noise background of the antibody [38].

Diverse methods of peak calling have been used by different programs. The most common strategy is to analyze distribution profiles to find clusters of reads that belong to the same peak. This strategy is used by different programs, including PIPE-CLIP [41], Pyicoclip [42], Piranha [43] and CLIPper [44], for all CLIP-seq protocol variants, and WavCluster [45] and PARanalyzer [46] for only PAR-CLIP data. PIPE-CLIP and Pyicoclip group the reads based on positional overlap, while Piranha, MiCLIP [47] and HITS-CLIP data analyses (http://qbrc.swmed.edu/softwares.html) bin on genomic portions by fixed size. On the other hand, CIMS focuses on the identification of read clusters containing mutated cross-linked nucleotides [25]. To discriminate enriched read clusters over the background, the peak-calling programs use different statistical models. For instance, PIPE-CLIP and Piranha use the zero-truncated negative binomial likelihoods, including also additional covariates to refine the peak detection, such as identification of cross-linked-dependent mutations or transcript abundance. On the other hand, Pyicoclip performs a background estimation implementing the modified false discovery rate procedure to determine which clusters of reads are significantly enriched in a list of genomic regions, by randomly placing the same number of reads within the region and iterating the process many times. MiCLIP [47] and HITS-CLIP data analyses use hidden Markov models (HMMs) to model the spatial dependency of the reads that map in the cluster. Finally, CIMS assesses statistical significance using a permutation-based model. Specific statistical models are used for PAR-CLIP.

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**Table 1. Number of reads obtained using different preprocessing and mapping tools on the in-house Ago2 HITS-CLIP data set from P19 stem cells**

| Replicate | # of reads before preprocessing | Preprocessing programs | # of reads after preprocessing | Mapping tools | # of reads after mapping |
|-----------|--------------------------------|------------------------|-------------------------------|--------------|-------------------------|
| 1         | 38 865 698                     | PRINSEQ                | 3 783 764                    | Novoalign    | 2 120 447 (56.0)        |
|           |                                | FASTX-Toolkit          | 933 985                      | STAR         | 1 142 926 (30.21)       |
|           |                                | CIMS                   | 2 467 666                    | Novoalign    | 1 355 710 (54.9)        |
|           |                                 |                        |                               | STAR         | 787 349 (31.91)         |
| 2         | 34.094.384                     | PRINSEQ                | 3 924 075                    | Novoalign    | 2 196 430 (56.0)        |
|           |                                | fastXtoolkit           | 957 572                      | STAR         | 1 311 574 (33.42)       |
|           |                                | CIMS                   | 2 584 470                    | Novoalign    | 1 425 553 (55.2)        |
|           |                                 |                        |                               | STAR         | 871 764 (33.73)         |
| 3         | 32.904.107                     | PRINSEQ                | 3 668 439                    | Novoalign    | 2 001 844 (54.6)        |
|           |                                | fastXtoolkit           | 888 186                      | Novoalign    | 1 152 648 (31.42)       |
|           |                                | CIMS                   | 2 359 084                    | Novoalign    | 1 264 546 (53.6)        |
|           |                                 |                        |                               | STAR         | 740 554 (31.39)         |

| Preprocessing | # of reads after mapping |
|---------------|--------------------------|
| Unique mapping (%) | Multiple mapping (%) | No mapping (%) |
| Replicate 1 | 2 120 447 (56.0) | 1 300 950 (34.4) | 34 565 (0.9) |
| Replicate 2 | 2 196 430 (56.0) | 1 321 658 (33.7) | 34 607 (0.9) |
| Replicate 3 | 2 001 844 (54.6) | 1 264 702 (34.5) | 35 261 (1.0) |
analysis. For instance, PARanalyzer uses a nonparametric kernel-density estimate classifier to identify RNA-protein interaction sites using T to C conversion rate and read density, while wavClusteR uses a two-step algorithm consisting of a nonparametric two-component mixture model and a wavelet-based procedure.

Importantly, different methods can give different results; thus, to help researchers in the choice of the right program for their peak-calling analysis, we recently performed a comprehensive, quantitative and qualitative comparative evaluation of four different publicly available programs for HITS-CLIP peak-calling step, including CIMS, PIPE-CLIP, Piranha and Pyicoclip, on four published Ago2 HITS-CLIP data sets [9, 48, 49] and one in-house Ago2 HITS-CLIP data set generated from P19 stem cells [24]. By tuning the programs in default parameters, we found that Pyicoclip outperformed the other programs in terms of sensitivity, positional accuracy, agreement with TargetScan motif discovery, we can cite MEME [56], GLAM2 [62] and cERMIT [63]. In addition to the sequence motifs, some programs take into account other sequence parameters, including secondary structure prediction, discovering therefore a structural motif that combines sequence composition with secondary structure. For such analyses, one may consider to use Zagoon [64], RNAcontext [65], MEMEris [66], PhyloGibbs [67] and Capri [68].

Motif discovery and other features

Following the peak calling, the analysis mainly focuses on the characterization of the RBP-RNA interactions, especially looking for possible binding sequence signature(s), using a candidate screening or a de novo motifs identification. For the candidate screening approach, programs like FIMO [54] can be used to screen peak sequences for the identification of known RNA-binding motifs, such as those from the database of Ray et al. [55]. If the user is looking for unknown RNA-binding motifs, a de novo motif identification could be performed. For this task, two main parameters should be calibrated before launching the analysis. The first parameter is the nucleotide length of the motif. Mitchell and Parker [56] recently showed that different RNA-binding domains bind in average a precise number of nucleotides. Thus, the length of the motif can be set according to the domain composition of the protein. The second parameter to take into account is the so-called ‘background sequences’ that can be used as negative template in which it is not expected to contain the enriched motif sequence(s). Two main strategies can be applied to select the appropriate background sequences: (i) to randomly scramble the CLIP-seq peak sequences; (ii) to define a set of sequences not bound by the protein of interest [57]. In the first strategy, a few constraints can be imposed to the background sequences, such as the dinucleotides frequency to avoid underestimation of false-positive rates in RNA prediction. As for the second strategy, instead, it is recommended that the background sequences possess the same size, dinucleotide frequency and the GC content of the target sequences used to perform the analysis. Besides these general options, each program allows to set its own parameters that may depend on the statistical/mathematical model used by the algorithm, including the distribution to model motif sites (i.e. the number of motif expected per sequence) and the threshold/score associated to the statistical model (i.e. P-value, enrichment score and probability). If successful, the final output of the de novo motif discovery analysis would be a list of sequences enriched in the CLIP-seq data set. On motif clusterization [58], these lists of enriched sequences can be represented as position weight matrices or position-specific affinity matrices that visualize the different affinity of the RBP for each sequence. Among the most popular programs for de novo motif discovery, we can cite MEME [59] and Homer [60]. Other programs that may be used are MatrixREDUCE [61], GLAM2 [62] and cERMIT [63]. In addition to the sequence motifs, alternative methods can be adopted by Clark et al. [74]; however, only the precomputed results for miRNAs expressed in 34 CLIP-seq data sets are available, but not the program. Finally, we have recently developed a novel method, called miRSShunter, that uses de novo motif search for an unbiased identification of miRNA-binding sites from Ago2 CLIP-seq data sets [24]. miRSShunter identifies any potential miRNA::RNA heteroduplexes for both canonical and noncanonical miRNA-binding sites, which involves portions of the miRNA sequence outside the seed or with seed-like binding, for searching for de novo motifs. Potential miRNA::RNA heteroduplexes are then ranked according to a heteroduplex score, which takes into account the following parameters of the heteroduplex: (i) free energy, (ii) the number of paired nucleotides, (iii) the number of paired nucleotides in the motif found, (iv) the number of paired nucleotides in the seed region and (v) the number of bulge nucleotides in the seed.

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Downstream analysis

The last step of CLIP-seq analysis involves functional characterization of the target RNAs identified to provide clues about the molecular function of the RBP(s) or the miRNA(s) of interest. Many programs/databases address this task, including GenTermFinder [75] and TopGO [76] to perform GO Term enrichment; GeneMania [77] to predict the function of a set of genes; STRING [78] and Cytoscape [79] to predict and visualize the protein interaction networks; GSEA [80] to determine whether a set of genes show similar expression differences between two biological conditions; and RAIN that integrates noncoding RNAs and protein–protein interaction networks [81]. Furthermore, the results from CLIP-seq analysis can be coupled with data sets from other genome-wide technologies, including RNA expression profile or alternative splicing.

Although not always routinely updated, many resources have been developed for the functional analysis of RBPs and miRNAs. For instance, miRonTop is an online Java Web tool that integrates DNA microarrays or high-throughput sequencing data to identify the potential miRNA target mRNAs by complementarity between the seed and the 3′ UTR sequences. The list of potential miRNA targets can be used to assess specific biological functions of miRNAs by performing Gene Ontology enrichment [82]. DIANA-mirExTra performs a combined differential expression analysis of mRNAs and miRNAs to uncover miRNAs and transcription factors that play regulatory roles between two conditions [83]. Finally, miRGator is a portal collecting high-throughput sequencing miRNA data integrated with target expression profiles [84]. This portal includes 73 deep-sequencing data sets on human samples from Gene Expression Omnibus [85], Short Read Archive (SRA) (SRA:http://www.ncbi.nlm.nih.gov/sra/), The Cancer Genome Atlas archives (http://cancergenome.nih.gov/) and several supporting programs. Among those programs, we mention miR-seq browser that provides short-read alignment with the predicted secondary structure of transcripts, read count and different features to study iso-miRs and miRNA posttranscriptional modifications.

Validation of CLIP-seq analysis

CLIP-seq experiments can be validated using different technical approaches by either candidate or genome-wide approaches [86]. For practical reasons, the candidate approach is feasible only to a limited number of targets, usually top-scored targets from statistical significance tests or identified by machine learning algorithms, or to a subset of targets with a particular biological relevance. The candidate approach can be performed to validate the direct interaction between the protein of interest and the target RNA(s) or the function played by the protein (or miRNA) on the target RNA(s). The interaction can be validated by a plethora of wet laboratory techniques, such as the in vitro electrophoretic mobility shift assay or RNA immunoprecipitation followed by either northern blotting or reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) from cell or tissue extracts. Functional validation may include knockdown/knockout or overexpression experiments on cells or tissues of the protein (or miRNA) of interest followed by RT-qPCR or northern blotting to check on the expression levels of the target RNA(s), or minigene assay to check on alternative splicing events.

The same functional validation can be also performed at the genome-wide scale assays, which may include RNA-seq or microarray experiments followed by an appropriate data analysis that depends on the function investigated. While the latter approach is more accurate and comprehensive, the cost can be higher.

Implication for miRNA-binding prediction and human pathogenesis

miRNAs are small noncoding RNAs of about 22 nts that associate to Ago2 to bind to RNA for degradation and/or translation block [16]. About 1000 miRNAs have been experimentally validated in human [87], which regulate many biological processes during physiopathological events and development [3, 4]. In this part of the review, we discuss the latest developments in the field of miRNA target prediction and mode of action in human pathologies made by the use of Ago2 CLIP-seq analysis.

Ago2 CLIP-seq data ameliorate miRNA target prediction

To date, the main miRNA target prediction programs take into account the following miRNA::RNA interaction features: (i) occurrence of perfect complementarity match between miRNA seed sequence and target miRNAs, (ii) sequence conservation of the target sequence across species, (iii) the free energy of the miRNA::RNA heteroduplex and (iv) the target site accessibility [88-91]. Even considering all these features, the rate of false positives and negatives is still high [70], indicating that more is needed to better predict miRNA target sequences. The presence of ~15 Ago2 CLIP-seq analyses performed in several cells or tissues and deposited in the Starbase database [92, 93] provides an important resource for a genome-wide investigation of the miRNA targeting features. Based on these studies, a second generation of miRNA target prediction programs has been developed. Although the second-generation programs seem to perform better than the first one, a major limitation for both of them is the lack of an accurate list of bona fide miRNA-binding sites to calculate true- and false-positive rates.

Here, we briefly describe the recent improvements of the prediction programs for miRNA-binding sites, based on CLIP-seq data (Table 2). Recently developed second-generation programs propose new models/parameters for the implementation of new algorithms. For instance, TargetSpy uses for the first time Ago2-CLIP-seq data to train a machine learning algorithm [100]. MIRZA develops a biophysical model through the parameterization of miRNA::mRNA target alignments and the free energy of the binding optimized using CLIP-seq data [101]. STarMir implements a logistic prediction models based on thermodynamic parameters of the miRNA::RNA heteroduplexes and the secondary structure features of the target miRNAs from CLIP-seq analyses [103]. MiRTar2GO is a rule-based machine learning approach to predict cell type-specific miRNA target mRNAs, which are ranked using validated binding sites from luciferase assay or Ago CLASH data sets [105]. Lu and Leslie [106] developed the program chimiciRic that uses a discriminative machine learning approach on Ago2 CLIP-seq and CLASH data to train a novel miRNA target prediction model. On the other hand, some of the first-generation miRNA prediction programs have been refined and updated thanks to Ago2 CLIP-seq analyses. For example, DIANA-micro-T-CDS [102] is an extension of the first-generation algorithm DIANA-micro-T [107] that uses a machine learning approach to identify the most relevant features of miRNA targeting from CLIP-seq data sets. Finally, the latest version of miRDB contains miRNA target prediction based on an updated version of the MirTarget computational model by including CLIP ligation (cross-linking and immunoprecipitation followed by RNA ligation) data in the training data set [104].
| Program name | Type of resource | Binding site position on mRNA | Type of binding site | Features | Reference | Web site |
|--------------|------------------|-------------------------------|----------------------|----------|-----------|----------|
|              |                  | 5' UTR | CDS | 3' UTR | Seed | NCSL | Conservation | Free energy | Accessibility | CLIP data |            |          |
| **First generation** |                |         |     |        |     |     |              |                |              |           |          |          |
| TargetScan   | WS               | No      | No  | Yes    | Yes  | No   | Yes          | No            | No          | No        | [93]     | http://www.targetscan.org/vert_71/ |
| PicTar       | WS               | No      | No  | Yes    | Yes  | SL   | No           | Yes           | Yes         | No        | [94]     | http://www.pictar.org/ |
| PITA         | WS/SA            | No      | No  | Yes    | Yes  | No   | Yes          | Yes           | Yes         | Yes       | [95]     | https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html |
| miRanda      | D/SA             | No      | No  | Yes    | Yes  | No   | Yes          | Yes           | No          | No        | [96]     | http://www.microrna.org/microrna/home.do |
| RNAhybrid    | WS/SA            | No      | No  | Yes    | Yes  | Yes  | No           | Yes           | No          | No        | [97]     | https://bibiserv2.ccb.jtu.edu.cn/rnahybrid?id=rnahybrid_view_download |
| **Second generation** |            |         |     |        |     |     |              |                |              |           |          |          |
| TargetSpy    | WS/D             | No      | No  | Yes    | Yes  | Yes  | No           | Yes           | Yes         | Yes       | [99]     | http://webdu.bio.wzw.tum.de/targetspy/index.php?search=true |
| MIRZA        | SA/WS            | No      | No  | Yes    | Yes  | SL   | No           | Yes           | No          | Yes       | [100]    | http://www.clipz.unibas.ch/downloads/mirza/ |
| DIANA-micro-T-CDS | WS/SA          | No      | Yes | Yes    | Yes  | No   | Yes          | Yes           | Yes         | Yes       | [101]    | http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index |
| STarMir      | WS               | Yes     | Yes | Yes    | Yes  | Yes  | No           | Yes           | Yes         | Yes       | [102]    | http://sfold.wadsworth.org/cgi-bin/starmir.pl |
| miRDB        | D/WS             | Yes     | Yes | Yes    | Yes  | No   | Yes          | Yes           | No          | Yes       | [103]    | http://www.mirdb.org/miRDB/ |
| miRTar2GO    | D/WS             | No      | No  | Yes    | Yes  | No   | Yes          | Yes           | No          | Yes       | [104]    | http://www.mirtar2go.org/ |
| chimiriC     | SA               | No      | No  | Yes    | Yes  | Yes  | No           | Yes           | No          | Yes       | [105]    | https://bitbucket.org/leslielab/chimiriC |

WS, Web server; D, database; SA, stand-alone software; CDS, Protein-coding sequence; NC, Noncanonical; SL, seed-like.
In addition to the features within the miRNA::RNA heteroduplex identified by CLIP-seq analyses, few reports showed that the binding activity of miRNAs is also modulated by RBPs that sit on the sequence surrounding miRNA-binding sites [108–110]. This has led to the concept of a sequence microenvironment surrounding miRNA-binding sites that can play an important role in regulating miRNA activity [89]. However, much remains to be explored about the use of RBP-binding motifs to improve the prediction of miRNA target sequences. A first step toward this direction was made by Incarnato et al. [111]. Briefly, the authors used Pumilio-binding motif to predict miRNA-binding sites within a distance of 100 nts. Validation of this analysis was carried out by RNA expression profile. We foresee that the full incorporation of RBP-binding motifs may pave the way to a third generation of miRNA target prediction programs.

**Application of Ago2 CLIP-seq in human pathologies**

Several publications reported miRNA targetome in different cells and tissues [12, 48–50, 110, 112–116]. Ago2 CLIP-seq experiments significantly contributed to our knowledge about the role of miRNAs in human pathogenesis. For instance, recently several reports use Ago2 CLIP-seq analysis to study the role of either host or viral miRNAs during viral infection [117]. Noteworthy, Kim et al. [118] combined Ago2 CLIP-seq and bioinformatics to identify miRNA targetomes of the human cytomegalovirus miRNAs during infection. This study reveals that viral miRNAs can regulate multiple pathways and cooperatively function with the host human miRNAs to promote viral replication [118].

Surprisingly for some human pathologies, such as cardiovascular diseases, despite the vast literature and the well-established roles of small RNAs in their pathogenesis [2, 119–121], genome-wide studies of miRNA regulatory networks are poorly developed. Indeed, only two Ago2 CLIP-seq studies have been performed in cardiovascular diseases, including one in the heart of transgenic mice overexpressing miR-133a and miR-499 [122], and the other one in the left ventricular cardiac tissue from six men with cardiomyopathy [123]. Interestingly, in ventricular tissue of patients with cardiomyopathy, about 4000 Ago2-binding sites that contain seed sequence complementarity for the highly expressed cardiac miRNAs have been identified. The authors deeply characterized the targetome of miR-133, known to be enriched in many pathological conditions of the heart and characterized new roles of miR-29 [123]. In particular, they found that miR-29 targets several mRNAs, including Ryr2, Serc2 and Junctin that are key regulators of sarcoplasmic reticulum Ca2+ in cardiomyocytes, PIK3R1 (p85-alpha) and Med13 that are involved in cardiomyocyte growth and metabolic signaling and Lama2 that plays a more general role in extracellular matrix composition of muscle cells. The authors speculate that these target genes suggest new roles for miR-29 in cardiomyocyte growth and calcium handling, which may have significant clinical relevance to cardiac hypertrophy and contractile dysfunction in cardiomyopathy patients. The novel aspect of these findings is also based on the fact that prior studies have focused on miR29 function in cardiac fibroblasts ignoring the cardiomyocytes [124]. Further preclinical and clinical investigations may strengthen these findings and eventually propose miR-29-based therapeutic approach to cure or prevent cardiopathies.

We foresee that Ago2 CLIP-seq experiments from biopsies of cardiovascular case-control studies and animal models will unravel the global role of miRNAs in the pathogenesis of cardiovascular disease and other human pathologies. A major limitation in the application of this approach is the limited amount of primary tissues derived from biopsies. Future technological advances to increase the depth of single-cell sequencing would overcome this limitation [125].

**Concluding remarks**

In this review, we discuss the recent computational developments of CLIP-seq analysis and highlight key points associated with each step, providing useful guideline to nonexpert users. We stress that a quality check of the data at each step is important to properly perform the analysis. While our manuscript was in revision, Uhl et al. [126] published a review on the CLIP-seq data analysis, which mainly focuses on the peak-calling step, whereas our review provides a more general point of view of the computational workflow. In addition, by addressing recent applications of CLIP-seq analysis for Ago2/miRNA target identification and prediction in physiological conditions or in human pathologies, our review provides practical examples of direct applications of this technique in biomedical fields. We believe that this review benefits scientists in RNA biology, gene expression fields and beyond.

CLIP-seq analysis provides a huge amount of data that often are not fully exploited by the researchers because of the lack of time or tools dedicated to perform/integrate different analyses. A further effort should be done to make these data available in a user-friendly format and resource databases to collect them. Some databases, such as StarBase [92], CLIPdb [127] and CLIPZ [27], collect raw data of CLIP-seq studies and provide peak sequences and/or coordinates. However, they are not always updated and use standard pipelines that may not be suitable for every RBP. Therefore, the development of integrated platform of CLIP-seq data analysis and databases could be a direction to be taken in the near future. In particular, these platforms should combine multiple software, such as pipelines covering multiple steps of data analysis or multiple databases including other high-throughput techniques as RIP-seq, ChiP-seq, RNA-seq and quantitative proteomics data, making the analysis faster and more comprehensive. On the other hand, improvement of the experimental protocol or conditions, such as quantification of the benefits of using replicates and/or control experiments, is needed to improve the reproducibility of the data and increase the efficiency of the data mining.

Overall, we have shown that CLIP-seq experiments associated to sophisticated bioinformatics analysis have become nowadays an essential instrument to gain insight into the direct regulatory network(s) of RBP-RNA interactions to address central questions of RNA biology and gene expression control in normal and pathological events of physiology or development. We foresee that progression in software development will take the stage in the near future to render the CLIP-seq analysis more integrated to other genome-wide approaches and more accessible to nonexpert users.

**Key Points**

- Recent developments in sequencing technologies and bioinformatics analyses enable us to handle many CLIP-seq samples simultaneously; thus, it is important to optimize bioinformatics pipelines that can facilitate the work of researchers to obtain unbiased and high-quality data.
- Despite great effort from researchers to streamline this CLIP-seq analysis, much remains to be improved on computational procedures.
In this review, we discuss the validity and the limitations of emerging programs for CLIP-seq analysis and the quality measurements currently available for specific tasks by providing concrete examples on an in-house Ago2 HITS-CLIP data set generated in stem cells.

We have focused the scope of this review in providing a valuable computational guideline for the bioinformatics analysis of the three main variants of CLIP-seq analysis, namely, HITS-CLIP, PAR-CLIP, and iCLIP.

We discuss how Ago2 CLIP-seq analyses have improved the miRNA-binding site prediction and the understanding of miRNA function in human pathologies.

Supplementary Data

Supplementary data are available online at BIB online.

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