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REVIEW

The methodological challenge in high-throughput profiling and quantifying microRNAs

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***Background*: MicroRNAs (miRNAs) play an essential role in various biological processes and signaling pathways through the regulation of gene expression and genome stability. Recent data indicated that the next-generation sequencing (NGS)-based high-throughput quantification of miRNAs from biofluids provided exciting possibilities for discovering biomarkers of various diseases and might help promote the development of the early diagnosis of cancer. However, the complex process of library construction for sequencing always introduces bias, which may twist the actual expression levels of miRNAs and reach misleading conclusions.**

***Results*: We discussed the deviation issue in each step during constructing miRNA sequencing libraries and suggested many strategies to generate high-quality data by avoiding or minimizing bias. For example, improvement of adapter design (a blocking element away from the ligation end, a randomized fragment adjacent to the ligation junction and UMI) and optimization of ligation conditions (a high concentration of PEG 8000, reasonable incubation temperature and time, and the selection of ligase) in adapter ligation, high-quality input RNA samples, removal of adapter dimer (solid phase reverse immobilization (SPRI) magnetic bead, locked nucleic acid (LNA) oligonucleotide, and Phi29 DNA polymerase), PCR (linear amplification, touch-down PCR), and product purification are essential factors for achieving high-quality sequencing data. Moreover, we described several protocols that exhibit significant advantages using combinatorial optimization and commercially available low-input miRNA library preparation kits.**

***Conclusions*: Overall, our work provides the basis for unbiased high-throughput quantification of miRNAs. These data will help achieve optimal design involving miRNA profiling and provide reliable guidance for clinical diagnosis and treatment by significantly increasing the credibility of potential biomarkers.**

Keywords: microRNA; next-generation sequencing; library preparation; bias

Author summary: Given the central importance of accurate quantification of miRNAs to molecular biology and clinical diagnosis, in this work, we reviewed recent findings on NGS-based quantification methods of miRNAs and discussed the possible deviations of each step. A series of optimization strategies were proposed to avoid or minimize such biases. In addition, combination optimization of various conditions during library preparation and eight commercially available low-input miRNA library preparation kits were described. Our work points out the problems in the existing library preparation process and summarizes possible optimization conditions that can be used for high-throughput profiling and quantifying microRNAs.

INTRODUCTION

MiRNAs are ~22 nt long non-coding RNA molecules that function by interacting with their mRNA targets in the cytoplasm or regulating the biogenesis and functions of non-coding RNAs (ncRNAs) in the nucleus [1–3]. They cycle in body fluids such as plasma, platelets, non-nucleated erythrocytes and nucleated blood cells, and of

course exist in tissues [4,5]. Although miRNAs account for only 0.5% to 9.2% of total RNA in human, they regulate various cellular activities, such as development, stem cell differentiation, growth/proliferation, cancer biology and stress response [6–8]. Almost all human diseases can cause significant dysregulation of miRNAs [9]. Therefore, it is a promising and exciting direction to use liquid biopsy of miRNAs as biomarkers for a broad range of human diseases, such as cardiovascular diseases, psychiatric diseases and cancers [10–14]. Valid biomarkers from biofluids achieve minimally invasive sampling and provide a more reliable method for early clinical diagnosis compared with traditional imaging diagnosis.

Accurate and effective profiling and quantification of miRNAs are prerequisites for understanding their roles in fundamental mechanistic pathways and discovering potential biomarkers and therapeutic targets. To date, the main platforms that exist for miRNA expression profiling are quantitative real-time reverse transcription PCR (qRT-PCR), microarray and NGS. Compared with qRT-PCR or microarray, NGS exhibits many advantages as follows: (1) the signal to noise ratio is better than microarray that based on hybridization technology; (2) it has a higher throughput than qPCR; (3) it offers the possibility to profile multiple samples in parallel; (4) it not only provides RNA length and sequence information at the single-nucleotide resolution but also allows to discover novel miRNAs and miRNA isoforms (isomiRs) without relying on prior gene annotations [14–16]. Consequently, NGS-based analysis provides many more possibilities and increasingly becomes the holistic trend for profiling small RNAs such as low abundance miRNAs in research and clinical diagnosis [17–20]. However, the major problem that cannot be ignored is the technical bias in NGS, which may severely distort the actual expression levels of miRNAs, namely, the expression levels of certain miRNAs appear to be overestimated, while others diminished or even undetectable although the sequencing depth has approached billions of reads [21–26]. Indeed, alternative library construction methods for NGS have inherent deviations and may generate significantly or completely different miRNA expression level profiles even with the identical starting material [14,27–30]. Moreover, a recent study reported that less than half of detected human miRNAs were quantified within 2-fold of expected value; however, many of the other remained a significant distortion even with commercially available small RNA sequencing kits [16]. These data indicate that miRNA sequencing remains challenging despite various improvements.

Here, we first systematically summarize recent researches that indicate various sources of bias in high-throughput profiling and quantifying miRNAs process and that optimize the NGS-based techniques and discuss from each step of miRNAs library preparation. Secondly, we report several representative multi-condition optimization methods. Finally, we describe the performance of commercially available low-input miRNA library preparation kits.

PREPARATION OF MIRNA SEQUENCING LIBRARY

NGS-based methodologies that widely are applied to study miRNAs in recent years greatly are expanding our understanding of the complicated biological processes, and we often accomplish this method by constructing sequencing libraries of miRNAs. Currently, preparation of NGS libraries of miRNAs starting with RNA samples generally involves (1) ligation of the oligonucleotide adapter to the 3′ end of RNA sample to provide a primer-binding site for subsequent reverse transcription (RT) and PCR amplification; (2) ligation of the 5′ adapter to the 5′ end of miRNA/3′ adapter ligation product; (3) removal of adapter dimer; (4) synthesis of cDNA by reverse transcription (RT) using a primer complementary to the 3′ adapter sequence; (5) PCR amplification that specifically enriches miRNAs with adapters on both ends; (6) library purification by size selection for sequencing. Previous studies indicated that almost all of the above steps are potential sources of bias [22,23,31–33]. Consequently, the sequencing results may not reflect the actual expression levels of miRNAs, especially those who already low in expression levels. In this section, we discuss the deviation issue in each step during constructing miRNA sequencing libraries and propose various strategies to optimize them.

Input RNA sample

In addition to technologies used in each step of library preparation, the quality and quantity of input RNA are responsible for the bias in miRNA read frequencies during NGS-based techniques [16,18,22,23]. Therefore, extracting high-quality RNA is the first step for successfully achieving unbiased profiling and quantification of miRNAs. However, the small size of miRNAs and the complexity of the sample, such as plasma with low levels of miRNAs, high levels of contaminating proteins and RNases, make miRNA extraction particularly challenging. The widely employed guanidium thiocyanate/phenol-chloroform RNA isolation strategies favor separation of specific miRNA species, which indicates that some low levels of miRNAs are at risk of being lost [34–36]. Application of carriers, such as yeast tRNA, glycogen and bacteriophage MS2, is a benefit for

improving RNA extraction efficiency [35,37,38]. Notably, the amount of carrier is critical to miRNA yield because high carrier concentration may cover the amount of target miRNAs and affect the accuracy of quantification and the quality of analysis [39]. Considering the ease of use and time input, some researchers choose appropriate commercial kits to extract RNA for library preparation [18,40]. By comparing six different commercial kits for miRNA isolation from fresh or frozen ovine plasma, Wright *et al.* indicated that three better kits are miRNeasy serum/plasma kit > miRNeasy serum/plasma advanced kit > Quick-cfRNA serum and plasma kit, while the PureLink RNA, Isolate and Monarch total RNA kits are not suitable for plasma miRNA studies [41].

In addition, based on our experimental experience, repeated freezing-thawing and RNases introduced by the operating environment usually cause miRNA degradation, which will affect the downstream steps of high-throughput sequencing.

Adapter ligation

The addition of adapters to both ends of each miRNA is the most widely used method to generate suitable terminal sequences for sequencing [42,43]. However, the 3′ and 5′ adapter ligation reactions catalyzed by phage-derived T4 RNA ligases exhibit nucleotide-based preferences, which are defined as the most significant contribution in introducing biases throughout the sequencing process and the read frequencies with errors even up to 1000-fold for some miRNAs [22,23,32,44–53]. Such disadvantages significantly hinder the utilization of high-throughput sequencing of miRNAs. Therefore, the optimization of this step is crucial for NGS analysis of miRNAs. Currently, we are aware of two ways in this direction, including improving adapter design and optimizing ligation conditions.

A critical concern in the ligation reaction is the formation of side products, which interfere with our discovery of the actual expression levels of the target miRNAs. Many data have indicated that reasonable adaptors can improve such a phenomenon, so it is of importance to design adapters [14,22,47]. Firstly, chemically synthesized 3′ adapters is generally designed with a 3′ amino-linker, a 3′ dideoxycytosine (ddC) or a fluorophore as the blocking element [17,22,24,47,54,55]. This 3′ end modification prevents the formation of undesired circularization and adapter concatenation at the step of linking the 3′ adapter to 5′-p miRNAs when using T4 RNA ligase [22]. Secondly, the 3′ adapter needs to be enzymatically adenylated at its 5′ end [17,47,54,55]. Adenylation of 3′ adapter allows performing ligation in the absence of ATP, thus avoiding self-circularization and self-polymerization of miRNAs [47]. However, several studies found that the circularization and concatenation of 5′-p RNAs are still competed with 3′ adapter ligation in ligase-catalyzed reaction even in the absence of ATP because of the rapid reversal of donor adenylation [22,31]. Furthermore, the specific RNA-adapter combinations at both ligation steps resulted in significant biases, and increased variability at the ligation junction of adapters could help to minimize distortion by enhancing RNA ligation yield [31,46]. Therefore, the 3′ adapter can be optimized by adding a randomized nucleotide fragment at the 5′ end to largely eliminates the bias caused by sequence preferences [17,24,28,45,46,48,56–61]. In addition, Fuchs *et al*. revealed that the adapter with an internal random region could also achieve this purpose and allows precisely determining the termini sequence of miRNAs [24]. Another alternative is the addition of a nucleotide homopolymer sequence at the 3′ end of miRNA sequence catalyzed by *E. coli* poly(A) polymerase instead of adding a precisely defined 3′ adapter [57], which circumvents deviation and adapter dimer contamination induced by adapter ligation. However, this method may lose any isomiRs either with 3′-(A) nucleotides.

Although DNA-based 3′ adapter sequence performs well when ligating to RNAs, the 5′ adapter typically is an RNA fragment that considered as preferred for RNA 5′ end ligation reaction [17,22,24,54,55,62]. Similar to the 3′ adapter, the 5′ adapter is improved with the following points: (1) with a blocking molecule at 5′ end, and (2) with a random region adjacent to the ligation junction [17,54]. Furthermore, recent studies indicated that secondary structures of adaptor and RNA have effects on ligation efficiency, and the 5′ adapter with a region complementary to the miRNA/3′ adapter ligation product can promote the formation of structures favorable for ligation [22,24]. Moreover, the addition of a unique molecular identifier (UMI) to each RNA sequence not only allows efficient counting of the absolute number of individual molecules but also reduces the ligation bias by randomizing the adapter sequences adjacent to the ligation junction [24,63]. Fishman *et al*. further verified this conclusion using UMIs provided by eight random nucleotides contiguous to 4 nt RNA barcode [40]. It is worth noting that the 5′ adapter used in this work is composed of a DNA fragment that with 4 nt RNA barcode at its 3′ end.

Instead of solely modifying the adapter mentioned above, some other studies sought to suppress bias by optimizing various ligation conditions. Here the choice of ligase as one of the possible sources of bias is discussed first. Currently available ligases mainly include four: T4Rnl1, T4Rnl2, truncated T4Rnl2 and the

mutant of truncated T4 Rnl2 (truncated T4 Rnl2 K227Q) [42,47,64]. By comparing the effects of T4Rnl1 and two Rnl2 variants on the 3′ adapter ligation reaction, Hafner *et al.* found that T4Rnl2 variant generated far fewer side-products than T4Rnl1 [22], which indicated that T4Rnl2 variant was the better choice. Moreover, Song *et al.* reported that truncated T4 Rnl2 K227Q was a better choice than truncated T4Rnl2 because the latter introduced a large number of background products [47].

Improving ligation efficiency up to near 100% for all miRNAs can significantly reduce the bias in the ligation step. For example, the RNA cloning method developed by Zhang's group achieves accurate quantification for a broad spectrum of miRNAs with less than a two-fold deviation from the anticipated value by guaranteeing ligation efficiencies of over 95% at both 3′ and 5′ ligation steps [17]. In this study, Zhang *et al*. described an optimized ligation strategy using adapters with a randomized two-nucleotide, a high concentration of polyethylene glycol (PEG) 8000 (concentrations of PEG 8000 at 3′ and 5′ adapter ligation are 10% and 20% respectively) and a thermostable DNA/RNA ligase or a 5′ splint adapter [17]. The application of PEG 8000, a macromolecular crowding agent, has always been an approach of great interest to researchers because it facilitates ligation reaction and has the most dramatic effect on the ligation efficiency among all the reaction parameters [47,57–59,65,66]. Higher concentrations of PEG 8000 are more suitable for ligation reactions. Song *et al.* revealed an optimal concentration range of PEG at 15% – 25%, while different optimization methodologies and designs would significantly affect the determination of the optimal PEG concentration [47]. Recently Kim *et al.* further optimized the concentration of PEG 8000 and found that 20% PEG 8000 in 3′ and 5′ adapter ligation steps minimizes the ligation bias in the presence of randomized adapters [28]. The thermostable RNA ligase, however, has not been observed to affect reducing bias in two subsequent studies [47,49].

Moreover, Hafner *et al.* indicated that incubation temperature and time in the ligation reaction are more critical parameters influencing ligation efficiency than the choice of ligases, and recent research suggested that 25°C was optimal for truncated T4 Rnl2 K227Q [22,47]. Not surprisingly, ligation efficiency is also affected by adapter concentration and ligase amount, which are different in the presence or absence of total RNA [47].

The ribosome profiling method of circularizing the first cDNA strand and then re-linearizing it at a different nucleotide can avoid the 5′ ligation step, which brings a lot of inspiration to researchers [53]. However, the circularization enzyme typically used has been shown to introduce bias [67]. Recently, Lama *et al*. reported an improved library construction method named Coligo-seq by adding a single ribonucleotide between the sequencing primer binding sites, and TS2126 Rnl1 and RNase A were introduced in the ribosomal profiling method [68], enabling that a variety of 5′ modified RNAs were sequenced and none step introduces strong bias. In addition, a new single-adapter ligation and circularization-based method is used to optimize the ligation step of library preparation of small RNA, and this strategy improves the accuracy of sequencing results and allows for the detection of a wider variety of miRNAs relative to other methods [50].

Removal of adapter dimer

In ligation processes, adapters are general excessive to maximize ligation efficiency. Unfortunately, this results in undesirable adapter dimer contamination because the free adenylated 3′ adapter is prone to form a dimer with subsequently added 5′ adapter. The products amplified from the adapter dimer might dominate the final sequence read when the concentration of input miRNA sample is low [14,69]. Besides, the efficient separation of miRNA products from adapter dimers-induced contaminations is challenging because of their similar sizes. Therefore, it is essential to remove those non-ligated adapters to minimize the formation of adapter dimers.

The denaturing polyacrylamide gel (PAGE), based on nucleic acid size selection for fragment separation, permits the separation of sequences between 2–500 bp with length differences as small as a single nucleotide [70]. Therefore, it allows for removing free adapters in the library construction of miRNAs [17,27,28]. However, the denaturing PAGE-based protocol not only is time-consuming and cumbersome but also results in a significant loss of the material. For some samples with low miRNA levels such as plasma, this method is not even feasible.

Compared with gel purification, the solid phase reverse immobilization (SPRI) magnetic bead, based on a non-specific reversible binding of nucleic acid molecules to magnetic beads coated with carboxyl groups, provides a more simple, rapid and efficient size selection method [71,72]. However, it is not capable of distinguishing nucleotide fragments between less than 100 nt. The instruction of SPRIselect (Beckman Coulter B23318) indicates that SPRI magnetic bead technology used for highly efficient miRNA enrichment just achieves an average enrichment efficiency of 61.63%. Recently, Fishman *et al*. established a modified SPRI that allowed separation of nucleotides shorter than 100 nt differing by as few as 20 nt in length by adjusting the concentrations of two crowding agents, isopropanol and

polyethylene Glycol (PEG) [40]. This improvement dramatically promotes the application of SPRI magnetic beads in the preparation of miRNA libraries.

Moreover, the locked nucleic acid (LNA) oligonucleotide that complementary to the ligation junction of the adapter dimer can reduce adapter dimers [73]. It specifically hybridizes to the adapter dimer by base pairing and extends into the 3′ adapter sequence, which blocks the annealing of primer in the reverse transcription step, thus excluding the adapter dimer contamination in the sequencing library. Vigneault *et al*. reported a similar method, in which miRNA/3′ adapter ligation products first hybridize with RT primer before ligating to the 5′ adapter; meanwhile, the free 3′ adapter forms a less-reactive double-stranded structure by annealing with the RT primer [69]. The widely used commercial kit, NEBNext® Small RNA Library Prep Set for Illumina, also use this method to prevent the formation of the adapter dimer. Another similar approach is the application of blocking oligo, a DNA fragment that complementary to the 3′ adapter and blocked at both ends, added after 3′ adapter ligation but prior to 5′ adapter ligation to prevent the generation of adapter dimer [40].

Phi29 DNA polymerase is a multifunctional enzyme. Previous studies indicated that phi29 DNA polymerase catalyzed two synthetic reactions and possessed pyrophosphorolytic and 3′ to 5′ DNA exonucleolytic activities [74,75]. Moreover, Lagunavicius *et al*. showed that the same enzyme also had a divalent metal ions dependent 3′ to 5′ RNase activity with the hydrolysis efficiency of single-stranded (ss)RNA was about 10-fold lower than that of ssDNA [76]. Phi29 DNA polymerase degrades ssRNA by gradually releasing a single nucleotide from the 3′ end of the nucleotide sequence, and this behavior will stop at the boundary of the RNA-DNA hybrid [76]. However, most of the applications of phi29 DNA polymerase are focused on rolling circle amplification (RCA), multiple strand displacement amplification (MDA) and RNA-containing circular substrates dependent RCA [77,78]. No reports that use phi29 DNA polymerase to degrade single-stranded adapter in library construction have been observed. Indeed, there are many limitations in using this enzyme to degrade free adapters while retaining the target product, but it opens new opportunities in this direction.

RT

Hafner *et al*. showed that RT does not significantly cause sequence-specific biases [22]. Studies focused on optimizing this step when constructing the sequencing library are rare, and we only learn that UMIs can be considered for further optimization to minimize RT deviation [79].

PCR

PCR is a fundamental tool for constructing sequencing libraries, and almost all existing NGS-based methods involve this step [33,80]. However, since PCR quickly reaches the plateau rather than a linear process, it will distort differences in expression [40,81]. Moreover, PCR is known to be prone to bias when multiple templates are amplified in parallel, where the reasons are that the length and sequence of the fragment affect amplification efficiency and that base composition may lead to preferred template-specific amplifications [33,80]. In particular, the GC-content of sequences can seriously affect amplification results and introduce unexpected bias because cDNAs with high GC-content are less readily amplified [82,83]. In addition, PCR noise gradually accumulates with a higher cycle number [84]. This feature is unfavorable for input miRNAs with limited concentration, in which certain low-expression miRNA species may not be detected at low cycle number.

Due to the widespread application of PCR and the limitations mentioned above, many efforts have been made to optimize this process [80,84]. Aird *et al*. indicated that the thermocycler and temperature ramp rate played surprisingly major roles in introducing GC bias, and a longer denaturation time, a DNA polymerase of AccuPrime Taq HiFi and lower annealing and extension temperature could be used to reduce amplification bias caused by GC content [85]. Several other studies show that Kapa HiFi and Herculase II Fusion enzymes are highly recommended [80,84]. So simply avoiding the use of certain polymerase according to the purpose of each experiment can dramatically reduce bias. Moreover, UMIs mentioned above not only reduce the ligation and RT bias but also correct PCR-induced artifacts and significantly reduce amplification bias [40,63,86]. Fu *et al*. indicated that the incorporation of UMIs into sRNA-seq procedure increased the data reproducibility and decreased background noise, which enabled accurate quantification of miRNAs even with a wide range of input RNA amount and number of PCR cycles [87]. The pre-amplification (PreAmp) reported recently is also a good choice for library preparation of miRNAs because it dramatically increases target miRNA abundance while minimizing the PCR bias [88,89]. Okino *et al.* developed a novel PreAmp reagent with an engineered DNA polymerase that improves amplification capacity and efficiency, thus resulting in less PCR bias and achieving accurate quantification even when the cycle number of PCR is up to 14 [88]. Instead of the traditional PCR method, linear

amplification is used to develop the pre-amplification procedure [90]. Since the linear amplification generates only a single target copy in each cycle, the template copy number increases in a linear fashion. This amplification form avoids the bias caused by the exponential amplification. However, this strategy still needs further optimization [91].

Touch-down PCR is more sensitive and special than the PCR with the same annealing temperature throughout the reaction [92]. Recent data indicates that touch-down PCR has been applied to establish the efficient full-length cDNA amplification strategy [93], which provides us with a possible direction that we can try to reduce PCR deviation in high-throughput sequencing of miRNAs.

Product purification

This step is essential for obtaining high-quality sequencing results, as purified products allow an increased sequencing depth, reducing costs and analysis complexity. The principle of product purification is usually based on the sequence size to separate the target product. Currently, the common method for product purification is non-denatured PAGE [17,28,55]. The SPRI magnetic bead strategy mentioned above and AMPure XP bead can also be used for PCR product purification [18,40]. However, it should be noted that AMPure XP beads can only bind nucleic acid fragments larger than 100 bp.

SEQUENCING PLATFORM

Commonly used sequencing platforms include Illumina and Ion Torrent sequencing. The accuracy of the Illumina platform is as high as 99.9%, while the latter is 98% [94]. Many studies have confirmed that the sequencing platform contributed little to the bias reported [14,23].

COMBINATORIAL OPTIMIZATION OF LIBRARY PREPARATION

Considering that many steps are possible sources of bias in cDNA library preparation, researchers prefer to use multiple optimizations to establish high-quality sequencing libraries that ensure high-throughput profiling and quantifying microRNAs. Here we introduce the following representative combinatorial optimization strategies.

Nguyen *et al*. developed a method termed as target-enrichment of sRNAs (TEsR) [54]. TEsR ensures the detection of rare sRNAs and the availability of multiple forms of sRNAs by introducing biotinylated RNA probes and optimized adapters and ligases [54]. However, it is not appropriate for studying novel sRNAs because the sequence information of the targeted sRNAs is necessary for designing the capture probes.

Quantitative small RNA sequencing (QsRNA-seq) is a method developed for preparing sRNA libraries for high-throughput sequencing, which allows very accurate, comprehensive quantification of miRNAs using optimized SPRI and 5′ adapters with UMIs consisting of eight random nucleotides [40,95]. In this protocol, the usage of modified SPRI technology enables simple and reliable separation between different nucleotides within 100 nt based on fragment size and analysis of smaller quantities of the input RNA compared to the PAGE-based method. By comparing the two reactions with the same conditions except for the presence or absence of UMIs, Fishman *et al*. indicated that the UMI, an 8 nt random sequence, not only used to determine absolute numbers of molecules but also significantly reduce ligation bias and subsequent PCR bias; the 4 nt barcodes at the 3′ end of 5′ adapter allow multiple samples to be processed simultaneously [95]. Simultaneously, applying a blocking oligo avoids the further formation of dimers and defective products [95].

Small-seq, a ligation-based method, is initially established for single-cell small RNA sequencing [55]. Compared with the traditional approach that uses rRNA pulldown removal strategy, the use of oligonucleotide masking of ribosomal RNA (rRNA) avoids the interference from the highly abundant 5.8S rRNA; the lambda exonuclease reduces the formation of adapter dimers by removing the free adapters; the UMI located at the 5′ adapter enables the removal of bias in PCR amplification and quantification of small RNA molecules; however, the bias still exists at the end of 3′ adapter [55].

In the accurate quantification by sequencing (AQ-seq) protocol, combinatorial application of randomized adapters containing four degenerate nucleotides and 20% PEG at both 3′ and 5′ adapter ligation reactions significantly improves miRNA quantification and allows detection of low abundance miRNAs that undetected in the traditional sRNA-seq method and ensures reliable definition of the terminal sequences of miRNAs [28].

In addition, Barberán-Soler *et al*. developed a novel method named RealSeq-AC, in which the use of a single adapter generated by incorporating both standard adapters at the 3′ end ligation step and the circularization of miRNA-adapter ligation product via intramolecular ligation provides the best accuracy for miRNA quantification among NEBNext, TruSeq, NEXTFlex, QIAseq SMARTer and RealSeq-AC [50]. A blocking molecule is also designed to inhibit adapter circularization by ligating to the 5′ pre-adenylated end of the unligated adapter, which allows for gel-free purification

and ensures a higher reproducibility with as low as 1 ng total RNA sample in miRNA detection [50].

COMPARISON OF COMMERCIALLY AVAILABLE LOW-INPUT MIRNA LIBRARY PREPARATION KITS

Recently, many commercial kits specifically for library preparation of low-input miRNAs have appeared on the market (Table 1). Many studies have focused on comprehensively evaluating the performance of these kits to achieve the optimal choice when designing experiments about miRNAs [16,30,57,79,96,97]. Anna *et al*. assessed systematic distortions during library preparation by using the universal reference miRXplore and found that SMARTer smRNA-Seq, Nextflex and QIAseq show the very similar coefficient of variation (CV) (~1.4) and almost identical miRNA percentage (45%–49% of synthetic miRNAs detected) within 2-fold of the mean, while NEBNext exhibits a higher CV (~2.7) and a lower percentage (only 20%) within 2-fold [16]. The data obtained from human plasma, human serum and murine brain tissue showed that Nextflex and QIAseq performed best for enriching miRNA mapping reads in biofluids and tissues [16]. Cloelia *et al.* further reported that the standard NEXTflex protocol was the best choice in human miRNAs, while SMARTer and CATS kits that based on ligation-free protocols performed relatively poorly in biological samples because of the significant generation of side products covered the advantages of owning the lowest levels of bias [57]. Other studies on the TruSeq, NEXTflex and NEB kits also show that the NEXTflex exhibits the best performance [98–100]. In addition, A recent study compared six commercially available low-input miRNA library preparation kits, including TailorMix, CleanTag, QIAseq, CATS and Small RNA-Seq Library Prep Kit (Lexogen GmbH), and concluded that the QIAseq performed best, the TailorMix kit closely followed [99]. In summary, the performance of the three highly recommended kits are sorted as follows: Nextflex ≈ QIAseq > TailorMix.

CONCLUSION

The ability to high-throughput profiling and quantifying miRNAs is of importance and certainly of interest to biomarker discovery and disease diagnoses but requires solving various bias from the sequencing process before application. Here, based on library preparation methods, we present various steps that induce severe bias in the relative expression levels of individual miRNAs and summarize available methods for minimizing bias (Fig. 1).

The step of adapter ligation is the most critical step resulting in serious deviations during library preparation of miRNAs, and a series of optimizations of adapter sequences and ligation conditions can minimize these deviations [24,45,47,49]. Moreover, based on experimental experience, the ligation efficiency of T4 DNA ligase is sensitive to the salt concentration and pH value in the reaction system. Thus, we must take the buffer in the reaction into account when determining the reaction conditions.

Currently, multi-optimization is the mainstream trend for library construction of miRNAs, and several combinatorial optimization methods introduced in this article can inspire the standardized process of miRNA library preparation. Moreover, comparing commercially available small RNA library preparation kits allows

Table 1 Summary of library preparation kit for small RNAs

Kit name	Features	Recommended input amount of RNA
Nextflex (Bioo Scientific/PerkinElmer)	RNA ligase-based ligation; randomized adapters; PEG; bead-based size selection	1 ng–2 μg total RNA or purified small RNA from 1–10 μg total RNA
NEBNext (NEB)	RNA ligase-based ligation; PEG; gel-based size selection (Pippon-Pre)	100 ng–1 μg total RNA
QIAseq (Qiagen)	RNA ligase-based ligation; bead-based size selection; UMI incorporated at RT step	1–500 ng total RNA
SMARTer (Takara Bio)	A ligation-free "tailing approach"; 3′ polyadenylation and 5′ template switching; gel-based size selection (Pippon-Pre)	1 ng–2 μg total RNA or small RNA
CATS Small RNA-Seq Kit (CATS)	A ligation-free "tailing approach"; 3′ polyadenylation and 5′ template switching	As less as 10 pg
Trilink Biotechnologies CleanTag Small RNA Library Prep Kit (CleanTag)	RNA ligase-based ligation; bead-based size selection	1–1000 ng
TailorMix microRNA Sample Preparation Kit Version 3 (TailorMix)	RNA ligase-based ligation; PAGE-based size selection	As little as 10 ng of total RNA
TruSeq (Illumina)	RNA ligase-based ligation	10–50 ng of purified miRNAs or 1 μg of total RNA

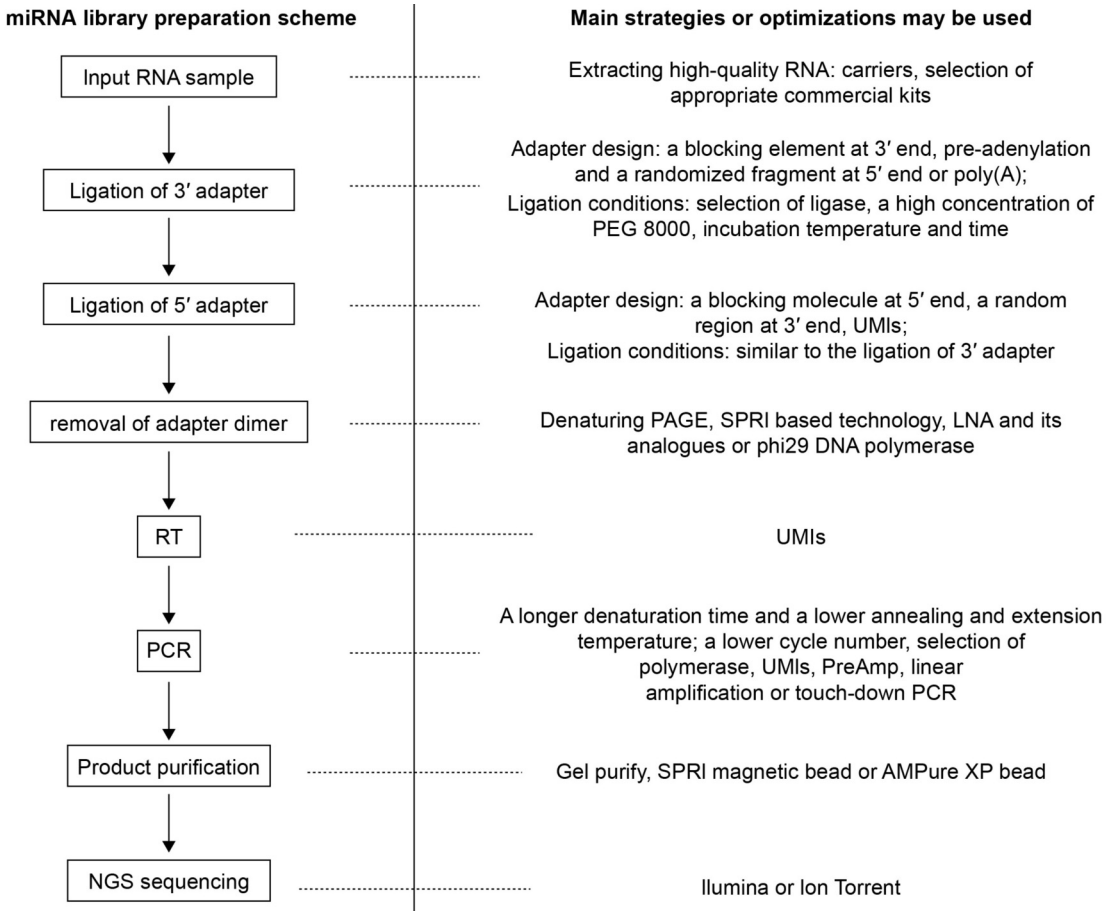

Figure 1. An overview of miRNA library preparation and the possible optimization strategies at every step.

researchers to make the best choice based on kit performance. However, it was showed that none could achieve accurate quantification of the relative expression levels of the majority of miRNAs, although various commercially available kits were used for sequencing library preparation of miRNAs [30]. Therefore, the establishment of rigorous standardization processes of miRNA high-throughput sequencing is urgent.

Collectively, our goal is to point out experimental strategies and techniques to avoid or minimize such deviations in miRNA expression levels so that improving the fidelity of NGS results. This work not only emphasizes the challenge of obtaining high-quality sequencing of miRNAs but also can serve as a guide for choosing the optimal strategy for miRNA library preparation. We believe that this will enable more accurate miRNA profiling and facilitate the development of reliable approaches for clinical diagnosis.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors Mengya Chai, Xueyang Xiong, Huimin Wang and Lida Xu declare that they have no conflicts of interests.

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