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Capture, Amplification, and Global Profiling of microRNAs from Low Quantities of Whole Cell Lysate

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that control gene expression at the post-transcriptional level via a complex regulatory network that requires genome-wide miRNA profiling to dissect. The patterns of miRNA expression at the genome scale are rich in diagnostic and prognostic information for human diseases such as cancers. This analysis, however, requires multi-step purification of RNAs from large quantities of cells, which is not only time consuming and costly but also challenging in situations where cell numbers are limited. In this study, we report direct capture, amplification, and library preparation of miRNAs from whole cell lysate without the need of pre-purification. As a result, it enables genome-wide miRNA profiling reproducibly with low quantity of cell samples (~500 hematopoietic cells). Specifically, we conducted a systematic investigation of two key steps – cell lysis for miRNA release and 3' adaptor ligation required for direct miRNA capture and amplification. The obtained expression profile not only distinguishes cell types but also detects individual miRNA alterations in closely related isogenic cell lines. This approach, which is substantially simple as compared to the standard methods because of elimination of the need for RNA purification, is advantageous for the measurement of low quantity samples.

Keywords

microRNA capture; microRNA profiling; low quantity sample analysis

INTRODUCTION

MicroRNAs (miRNAs) are a functionally important class of small RNAs of ~22 nt in length that regulate gene expression post-transcriptionally¹. The functions of miRNAs have been demonstrated across nearly all major domains of biology. Their expression patterns have

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been found to be highly informative to reveal distinct disease states such as human cancers². According to the biogenesis of miRNAs, primary miRNAs (pri-miRNAs), precursor miRNAs (pre-miRNAs), and mature miRNAs are simultaneously present in a live cell, and two different mature miRNAs can be made from the same pre-miRNA; this leads to significant heterogeneity of mature miRNAs to differentially and predominantly regulate the post-transcriptional processes³; therefore, it is highly desirable to perform unbiased amplification and profiling of the whole miRNA pool. The major approaches available for miRNA profiling include quantitative reverse transcription PCR (qRT-PCR) array, hybridization-based microarray methods, and high-throughput sequencing⁴. qRT-PCR array can be carried out in medium throughput¹. Due to high sensitivity of qRT-PCR and its large dynamic range, it has been extended to the measurement of one or several known miRNAs in single cells⁵. Hybridization-based microarray methods have high throughput, but lower specificity than qRT-PCR. Whole pool amplification (~1000 miRNAs) followed by unbiased microarray profiling or deep sequencing provides high accuracy in discriminating highly similar miRNA sequences, such as isomiRs¹, as well as the capability to detect unknown miRNAs. How to capture and amplify the whole pool of small RNAs including all miRNAs is a critical step toward reliable miRNA profiling in both basic and clinical miRNA research^{1,4}.

Although whole pool amplification has been widely demonstrated to capture messenger RNAs (mRNAs) and prepare whole mRNA pool amplicons and libraries from low quantities of cells or even single cells^{6,7}, it is not readily expandable to miRNAs due to several key differences between miRNAs and mRNAs. Moreover, mature miRNAs are short in length and do not contain poly(A) tails and thus cannot be incorporated into current mRNA processing and cDNA amplicon preparation protocols. Additionally, mature miRNAs are bound by Argonaute (AGO) proteins, which form a core component of RNA-induced silencing complexes⁸. Vast majority of mature miRNAs are highly stable^{8,9}, a property that has been attributed to the protection by the AGO proteins; thus, the loading of miRNAs into AGO increases the miRNA stability^{8,10}. Crystal structure analysis has further revealed that one of the AGO family proteins, AGO2, can tightly bind to a mature miRNA molecule and protect its ends^{11,12}. However, this also suggests that the cell lysis condition needs to be modified to release miRNAs from the AGO complex, which differs from the extraction of messenger RNAs.

Nowadays, the standard protocol for whole miRNA pool library preparation is based on a ligation-mediated amplification strategy, which involves sequential ligation of adaptor oligonucleotides on the 3' and 5' ends of miRNA molecules (before reverse transcription and PCR amplification, Figure 1)². One of the key steps is the ligation at the 3' end of miRNA (referred to as 3' ligation), which utilizes 5'-adenylated oligonucleotides (referred to as 3' adaptor) and mutant T4 RNA ligase 2 to avoid self-ligation of miRNAs¹³. Chemical-based adenylation of 3' adaptor is feasible, but very expensive. Alternatively, inexpensive biochemical adenylation of 5'-phosphorylated oligonucleotides has been described using T4 DNA ligase¹⁴, T4 RNA ligase 1¹⁵ or Mth RNA ligase¹⁶. However, biochemical adenylation is not complete, leaving fractions of un-adenylated but phosphorylated 3' adaptor molecules that can react with 5' adaptor to create adaptor dimers and reduce overall library preparation efficiency. The effects of a range of other parameters

including ligase type, amount, PEG concentration, adaptor concentration, incubation time, and reaction temperature on capture efficiency and bias have been studied to improve the capture efficiency and reduce the variability across different miRNAs¹⁷.

However, all these approaches require the use of purified total RNAs or purified fractions of small RNAs as the starting material; thus, a large number ($\sim 10^6$) of cells need to be used for the purification process to generate sufficient amounts of high-quality RNA preparations for small-RNA capture. This requirement poses significant challenges when the sample size is limited (e.g., <1,000 cells), making it difficult to apply these approaches for the profiling of rare cell populations or single cells. As such, to minimize the loss of miRNAs in the purification step and achieve miRNA profiling from low quantity or single cell samples, it is desirable to develop new protocols that can directly use whole cell lysate for 3' ligation and miRNA capture and to enable global miRNA profiling in low quantity cell samples.

Herein, we report direct capture and amplification of miRNAs from whole cell lysate and the global profiling of miRNAs from low quantity cell samples (<1000 cells). During the preparation of this manuscript, Faridani *et al.* reported single-cell sequencing of small-RNA transcriptome¹, which was also based upon the direct capture and amplification of small RNAs from whole cell lysate via a similar but not identical protocol. However, this was not a systematic study, and how the experimental conditions affected the performance was unclear. In this study, we present a systematic evaluation of various experimental parameters to improve the capture efficiency and amplification from whole cell lysate and rigorous validation with specific miRNA knock-out lines. Moreover, two major technical improvements were developed. First, the lysis protocol was improved to allow efficient release and direct capture of miRNAs without purification. Second, we devised a unique quenching approach to inactivate 5'-phosphorylated 3'-adaptor after the enzyme-based adenylation reaction. This study provides a new route towards global profiling of miRNAs from low-quantity samples, in particular, primary cells from clinical specimens, and has the potential to address the critical need for the examination of the role of miRNAs in intratumor heterogeneity and immune cell functional diversity; moreover, the proposed route can further be developed for single-cell genome-scale miRNA profiling.

MATERIALS AND METHODS

Cell culture

A murine BaF3 hematopoietic cell line was obtained from the American Type Culture Collection (ATCC) and has been previously reported¹⁹. BaF3 cells were cultured in an RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Life Technologies), 1% of (100×) pen/strep/glutamine (Life Technologies), and 3 ng ml⁻¹ of recombinant murine IL-3 (Peprotech). An NIH3T3 cell line was obtained from ATCC and has been previously reported¹⁹. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were visually inspected to confirm their expected morphology. BaF3 cells were tested to confirm their dependence on IL-3.

The miR-142 knockout BaF3 cells have been previously reported¹⁹. Briefly, the wildtype BaF3 cells were infected with an miR-142-3p reporter, wildtype Cas9, and an sgRNA against miR-142 (targeting sequence: cggagaccacgccacgcgcg). A clonal cell line was derived from these infected cells via FACS sorting on desired reporter level, which was confirmed to have a knockout status of the miR-142 locus.

Isolation of total RNA and preparation of cell lysate

For RNA isolation, the wildtype BaF3 cells or miR-142 knockout BaF3 cells were washed once with cold PBS (Life Technologies) and counted. For comparison of miRNA detection between total RNA and direct lysis, RNA extraction was performed with TRIzol (Life Technologies) from 100,000 cells following the manufacturer's protocol. Note that for qRT-PCR experiments, evaluating lysis and heating conditions, synthetic hsa-miR-371-3p (Integrated DNA Technologies) was spiked into the TRIzol lysate at 3.3×10^{-15} moles per sample before continuing RNA preparation. RNA pellets were suspended in 10 μ l RNase free water. For preparation of cell lysate for qRT-PCR, 100,000 PBS-washed cells were pelleted via centrifugation, and cell pellets were lysed by 5 μ l 1% Triton X-100, containing 3.3×10^{15} mol synthetic hsa-miR-371-3p. Before the qRT-PCR analysis, cell lysate was treated by heating when indicated. Preparation of cell lysate for small-RNA sequencing library was performed similarly, but synthetic hsa-miR-371-3p was not added. In addition, to avoid RNA degradation, RNase inhibitor (Ambion) was added at a 1:25 ratio to the lysis buffer.

qRT-PCR analysis of the miRNA expression

RNA samples or cell lysate samples were subjected to reverse transcription (RT) reaction using an miRNA reverse transcription kit (Qiagen) with a high-spec buffer. For RNA samples, 300 ng of total RNA was used. For cell lysate experiments, 5 μ l lysate was used as an input for RT. The qPCR analysis was performed via the Qiagen qPCR kit for miRNAs and specific primers for mmu: miR-142-3p, hsa-miR-371-3p, and miR-222-3p. We normalized the qRT-PCR data of miR-142-3p and miR-222-3p with that of hsa-miR-371-3p, and the latter was not endogenously expressed in the BaF3 cells. Normalized data reflected the detected miRNA expression relative to that of the spiked-in miR-371 control (and hence the starting cell number).

Preparation of 3' adaptors

A 5'-phosphorylated 3' adaptor was ordered from IDT and had the following sequence: 5'-p-TGGAATTCTCGGGTGCCAAGGCAA-3'-BioTEG. All bases were DNA bases. Pre-adenylation of 5'-phosphorylated 3' adaptor was performed following a reported protocol²⁰. Specifically, 1 nmole of 5'-phosphorylated 3' adaptor was mixed with 40 μ l 50% PEG 8000 (BioLabs), 10 μ l 1x T4 RNA ligase reaction buffer, and 5 μ l T4 RNA ligase 1 (Thermo Fisher EL0021) in a total volume of 100 μ l and reacted at room temperature overnight. Reaction mixture was heat inactivated at 65°C for 15 minutes before purification using the QIAquick nucleotide removal kit (Qiagen). Dephosphorylation was performed using antarctic phosphatase (NEB) by treating the products of the pre-adenylation reaction with 2 μ l of phosphatase in a volume of 60 μ l. Dephosphorylation was performed at 37°C for 2 hours before heat inactivation at 80°C for 5 minutes. Quality control was performed to resolve the reaction products through electrophoresis on 10% denaturing polyacrylamide

gels. Gels were prepared by adding 8.75 ml Sequel NE reagent (Part A, American Bioanalytical), 8.75 ml Sequel NE reagent (Part B, American Bioanalytical), and 140 µl 10% ammonium persulfate (Sigma Aldrich). Samples were prepared by adding equal volumes of 2x urea loading buffer (8 M urea, 50 mM EDTA, pH = 8.0, 0.05% bromophenol blue, 0.05% xylene cyanole; all chemicals were obtained from Sigma Aldrich) and denatured at 80°C for 5 minutes. Electrophoresis was performed at 280 V for 1 hour. After electrophoresis, the gel was stained in 15 ml water containing 1.5 µl GelStar Nucleic Acid Gel Stain (LONZA) for 15 minutes.

Evaluating the efficiency of 3' ligation reaction

Synthetic miRNAs (sequence: 5'-p-GACCUCCAUGUAAACGUACAA) were purchased from Integrated DNA technologies (IDT). Herein, 5 pmole of synthetic RNA was ligated to freshly prepared 3' adaptor (pre-adenylated and dephosphorylated) using T4 RNA Ligase 2 Truncated KQ (NEB M0373S) at room temperature for 6 hours. The reaction mixture also contained the manufacturer supplied reaction buffer (final concentration of 1x) supplemented with 0, 0.5%, 1%, 1.5%, and 2% Triton or 2% SDS. Half of the reaction products were then inactivated via heat treatment at 80°C for 5 minutes and resolved on a denaturing polyacrylamide gel following the procedure described in the abovementioned section.

Small-RNA profiling library preparation

Total RNA or cell lysate was used for small-RNA library preparation. Herein, 500 ng of RNA or lysate from 100,000 cells was ligated to the prepared 3' adaptor (pre-adenylated and dephosphorylated) using T4 RNA Ligase 2 Truncated KQ (NEB M0373S) at room temperature for 6 hours. A quenching step to inactivate the unreacted pre-adenylated 3' adaptor was performed by adding the quencher dAdCdG dGdAdA dTdTdC dCdTdT dAdCdT AAA for 15 minutes. Alternatively, a size purification step was performed to purify the ligation products using 10% denaturing polyacrylamide gel. Moreover, 5' ligation reaction was performed with the following adaptor: GTTCAGAGTTCTACArGrUrCrCrGrArCrGrArUrC (Dharmacon), with rN denoting RNA bases. Then, 5' ligation reaction was performed with the T4 RNA ligase 1 (Thermo Fisher EL0021) in 10 µl volume for 6 hours at room temperature. An optional gel purification step was performed after 5' ligation using a 10% denaturing polyacrylamide gel. Reverse transcription was performed with the M-MLV reverse transcriptase (RT) (Invitrogen 28025–013) using the primer GCCTTGGCACCCGAGAATTCCA. The RT product was polymerase chain reaction (PCR) amplified for 31 cycles with Phusion DNA polymerase (NEB M0530) using a 3'-primer 5'-GCCTTGGCACCCGAGAATTCCA-3' and 5' primer 5'-biotin-GTTCAGAGTTCTACAGTCCGAC-3' (IDT). Barcoded small-RNA libraries were detected via a bead-based miRNA detection system using the Luminex machine².

RESULTS

Two-step cell lysis for effective miRNA release and detection in whole cell lysate

To determine an optimal strategy for direct miRNA profiling from whole cell lysate, we first used the BaF3 cells, a murine hematopoietic cell line, and engineered them to obtain a

constitutive expression of the red fluorescent protein mCherry. Upon testing them with 1% (V/V) Triton X-100 as the lysis buffer, we observed quick lysis of the cells within 1 minute at room temperature, as reflected by the loss of cell integrity via both light and fluorescent microscopy (Figure 2A).

We next determined whether mature miRNAs within cell lysate could be efficiently detected. In this regard, we used a poly-adenylation-based qRT-PCR approach that required access to free 3'-OH group on miRNAs⁸. To quantify the efficiency of mature miRNA detection, we measured the expression of miR-142-3p, a hematopoietic-cell-enriched miRNA, in BaF3 cells and compared cell lysate against total RNA extracted from the same number of cells using a conventional protocol (Qiagen). The BaF3 cells were treated with the lysis buffer, ranging from 0.5% to 2% in concentration (m/m), for 5 minutes. To control the variations in sample preparation and reverse transcription, we spiked in chemically synthesized hsa-miR-371-3p, a human pluripotency miRNA not expressed in BaF3 cells²¹, before cell lysis or RNA extraction (see methods). While the expression of miR-142-3p could be detected above the background (Figure 2B), as determined using a clonal miR-142 knockout cell line derived from BaF3 cells¹⁹, the signals from cell lysates were >10-fold lower than those obtained from the extracted RNAs. These results suggest that mature miRNAs cannot be efficiently detected in cell lysate via a simple one-step lysis procedure.

We reasoned that mature miRNAs were loaded onto the AGO proteins⁸, which could present a barrier for reactions of mature miRNA molecules in cell lysate. We further reasoned that addition of a heating step after cell lysis may lead to the denaturation of miRNA-associated protein(s) and allow effective detection of mature miRNAs. Indeed, brief heating of cell lysate at 75°C or 95°C yielded comparable levels of miR-142-3p and miR-222-3p detection to those obtained from the extracted RNAs from BaF3 cells (Figure 3A). To determine an optimal heating temperature, we examined a series of temperatures ranging from 25°C to 95°C. Consistent with the need for a heating step, low levels of miR-142-3p were detected at and below 37°C, with the detection increasing with the increase in the heating temperature (Figure 3B). We found that 75°C yielded the best signal, whereas 95°C resulted in slightly decreased signals possibly due to instability of RNA at high temperatures. Overall, we concluded that a two-step lysis protocol, involving Triton X-100-based cell lysis and a 75°C heating step, can allow efficient release and detection of mature miRNAs directly from cell lysates.

Facilitation of the capture of miRNAs via 3'-end enzymatic ligation through inactivation of the unadenylated 3' adaptors

As a key reagent in the 3' adaptor ligation process for miRNA library generation, the pre-adenylated 3' adaptor is critical and can be used in combination with the RNA ligase 2 mutant. To avoid issues related with the incomplete 3' adenylation reactions in biochemical pre-adenylation reactions, we devised a strategy to inactivate phosphorylated but unadenylated 3' adaptor molecules using phosphatase, and the resultant dephosphorylated 3' adaptor was unable to react with the 5' adaptor molecule. Specifically, we used Antarctic Phosphatase for T4-RNA-ligase-1-catalyzed pre-adenylated reaction products followed by heat inactivation of phosphatase and oligonucleotide purification. On resolving the reaction

products on high percentage denaturing polyacrylamide gels, we observed successful adenylation and efficient dephosphorylation of the 3' adaptors after 10 pmole of each sample was loaded onto each lane, such that >60% of the products were adenylated 3' adaptor molecules with undetectable levels of phosphorylated 3' adaptors (Figure 4A). These data support an improved protocol for the inexpensive adenylation of the 3' ligation adaptor.

Evaluation of the ligation reaction in the cell lysis buffer

To directly utilize cell lysate for adaptor ligation, it is important that the Triton X-100 lysis buffer does not inhibit ligation. We evaluated this biochemical reaction using synthetic small RNAs with the adenylated 3' adaptor, and different concentrations of Triton X-100 were added to the reaction mixture. Ligation reactions were then resolved on denaturing polyacrylamide gels. Figure 4B showed that up to 2%, Triton X-100 did not have a major negative impact on the level of successfully ligated products. These data further support the feasibility of performing small-RNA capture, amplification, and library preparation directly from whole cell lysate.

Capture, amplification, and profiling of miRNA from whole cell lysate

Under the abovementioned optimized lysis conditions and ligation conditions, we next performed small-RNA library preparation from whole cell lysate following the entire workflow shown in Figure 1. To assist the evaluation of the performance, the murine hematopoietic BaF3 cell line, a clonal BaF3 derivative cell line with miR-142 knockout (referred to as BaF3 KO), and NIH3T3, a murine fibroblast cell line, were utilized. The number of cells initially used was 100,000 per sample, and each cell line was analyzed in duplicate. After RNA capture, amplification, and library preparation, the miRNA expression was quantified using a microarray-like bead-based profiling system that directly measured the miRNA content within the small-RNA library based on hybridization to the predesigned probes². Unsupervised clustering analysis revealed distinct profiles between three cell lines (Figure 5A). Importantly, technical replicates were tightly clustered together, capable of distinguishing even the closely related cell lines BaF3 and BaF3 KO. In addition, the BaF3 KO cells were closely clustered with BaF3 cells, with the major distinction being miR-142-3p and miR-142-5p (Figure 5A and B), two mature miRNAs produced from the miR-142 hairpin. Further examination revealed good correlation between technical replicates (Pearson Correlation $R = 0.9885$) (Figure 5C). Overall, the abovementioned data indicate the successful small-RNA library preparation directly from whole cell lysate, and this approach yielded excellent reproducibility as well as specificity to distinguish closely related cell samples that differed by a single miRNA gene.

Profiling of miRNAs in low-quantity cell samples

To determine whether it was feasible to obtain informative miRNA expression profiles from a low number of cells, we directly generated lysates from 50, 500, 1500, or 5000 BaF3 cells as input for miRNA capture, amplification, and library preparation. Herein, two replicates were performed for each condition of cell number. Using a Luminex bead array profiling approach (with a total of 582 probes, see the Supplementary Tables 1 & 2), we selected a panel of ~70 miRNAs with high intensity from the data of 5000 BaF3 cells. This number

dropped to ~36 and ~23 miRNAs, for 1500 and 500 cells, respectively, but was still unambiguously detectable. Even for 50 BaF3 cells, ~10 of these miRNAs were detected with the same criteria (Supplementary Figure S1). Global unbiased analysis of miRNA expression patterns obtained from 1500 cells correlated well with those obtained from 5000 cells ($R = 0.962$) (Figure 6A). Further examination revealed that miRNAs, such as miR-142-3p, miR-let-7b, and miR-let-7c, enriched in BaF3 cells were well detected in the 5000- and 1500-cell samples. Although a dropout of miRNAs obtained from the 500-cell sample was observed, the correlation with those of the 5000-cell sample was still excellent ($R = 0.939$) and almost all signature miRNAs were successfully detected (Figure 6B). These results indicated that the informative miRNA profiles could be obtained from as low as 500 to 1500 cells directly from cell lysate.

DISCUSSION

To date, it is still difficult to perform unbiased global profiling of miRNA biomarkers from low quantities of cell samples, in particular, from those derived from clinical specimens. One of the limitations is the requirement of total RNA purification, which is not readily scalable and is incompatible with high-throughput biochemistry to capture, ligate, and amplify small RNAs. In this study, we demonstrated that by optimizing cell lysis condition and ligation workflow, the whole miRNA pool libraries could directly be generated from cell lysates. While we still used enzymatic 3' and 5' ligation to select small RNAs including miRNAs for reverse transcription, amplification, and whole miRNA pool library generation, we made several key improvements that enabled the direct use of whole cell lysate and its application to low-quantity cell samples. Unlike cell lysis and capture of mRNAs, a heating step was required in our protocol to release miRNAs, and a systematic study on the effect of temperature was presented. We also devised an inactivation process to remove the potential negative impact of phosphorylated but unadenylated 3' adaptors to increase the efficiency of reactions toward the whole miRNA pool amplification and library preparation.

Using our approach, we reproducibly obtained miRNA expression profiles from small-RNA libraries generated directly from whole cell lysate. The expression patterns faithfully distinguished between hematopoietic and fibroblastic cell types. In addition, unsupervised clustering analysis of the expression profiles was able to detect the subtle differences between isogenic cell lines, namely BaF3 and BaF3 KO, which differed by a single miRNA (miR-142) gene. These data supported that reproducible and informative miRNA expression profiles could be obtained directly from whole cell lysate. By titrating the cell number, we showed that global profiling of miRNAs gave highly consistent results down to 1500 or 500 cells. At the level of 50 cells, we still detected meaningful expression of select miRNAs, but with much fewer detected miRNAs. Considering that abundantly expressed miRNAs in cell lines have been estimated to be ~2000 to 10,000 molecules per cell²², we estimated the detection sensitivity of our method to be ~100,000 to 500,000 miRNA molecules in a 5 μ l lysis buffer. We speculate that if the cell number is low, due to low substrate concentration, biochemical reactions such as T4 ligation reaction become less efficient. In addition, technical loss during each experimental step may accumulate to limit the overall efficiency. Note that these experiments were conducted on a fast-proliferating BaF3 cell line (with doubling time less than 12 hours), and fast-proliferating hematopoietic progenitor cells

tended to have lower levels of the global miRNA expression²². It is thus possible that our method can be extended to even lower numbers of cells, for example, if the cells are post-mitotic or have slower cycling speed.

Our method can potentially be adopted for high-throughput generation of miRNA libraries from large numbers of samples by eliminating the sophisticated and inconvenient total RNA purification step. Since our samples only require lysis and heating steps before further processing, the RNA extraction time is reduced from more than 2 hours to less than 10 minutes as compared to that of the conventional protocols. We further demonstrated an improved method for the preparation of the adenylated 3' adaptor sequence. This improvement allows future inexpensive preparation of barcoded 3' adaptors, which can enable multiplexed library preparation, and a large number of samples can be pooled for deep sequencing. Furthermore, by eliminating the total RNA extraction and purification step, our method can potentially be integrated in a microfluidics system to perform single-cell level miRNA capture, amplification, and whole pool library preparation with further reduction of the reaction volumes.

Although the data shown herein has been obtained using an inexpensive bead-based detection system for mature miRNAs, the same library can be analyzed through next-generation sequencing as the adaptor sequences are compatible with Illumina flow cells. We have previously demonstrated this cross compatibility of the same library for sequencing and microarray-based detection⁸. Since the library prepared can also contain small RNAs other than miRNAs, this suggests that our method of miRNA library preparation can be utilized toward directly profiling other functional small RNAs from whole cell lysate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Experimental workflow – cell lysis, miRNA release, capture via 3' adaptor ligation, followed by 5' adaptor ligation for PCR amplification and library preparation

3' adaptor is pre-adenylated at the 3' end before ligation with miRNA catalyzed by T4 RNA ligase II (w/o ATP). The PCR adaptor coupling is completed via ligation to 5' adaptor using T4 RNA ligase I (with ATP). This workflow is used to amplify miRNAs and quantify the expression globally at the genome-scale. Moreover, two optional size selection processes were performed using gel purification after each ligation step and before amplification. The two steps in orange highlight the major improvements reported in this study.

Figure 2. Cell lysis and effect of lysis buffer concentration on the qRT-PCR detection of miR142
 (A) BaF3 cells stably expressing mCherry as a fluorescence marker were lysed with 1% Triton buffer in 30 seconds. Representative optical (Phase Contrast) and fluorescence images are shown before and after lysis. (B) BaF3 cells (WT) or BaF3 cells with miR-142 knockout (KO) were subjected to RNA preparation with TRIzol or cell lysis with lysis buffer containing the indicated concentrations of Triton. Synthetic miR-371 was spiked in to control experimental variation. miR-142-3p and miR-371-3p levels were determined via qRT-PCR. The relative detection levels of miR-142-3p are shown in log scale to reflect quantification from the same fraction input. N = 3. Error bars present the standard deviation.

Figure 3. Effect of post-lysis heat treatment on the detection of miR-142 and miR-222 by qRT-PCR

(A) BaF3 cells (WT) or BaF3 cells with miR-142 knockout (KO) were subjected to RNA preparation with TRIzol or cell lysis with lysis buffer containing 1% Triton. Synthetic miR-371 was spiked in to control experimental variation. Levels of miR-142-3p, miR-222-3p and miR-371-3p were determined by qRT-PCR after heat treatment post cell lysis. The relative detected levels of miR-142-3p and miR-222-3p are shown in log scale to reflect quantification from the same fraction input. (B) Relative levels of miR-142-3p were determined by qRT-PCR of BaF3 cells (WT) with heat treatment at the indicated temperatures for 5 minutes. N = 3. Error bars present the standard deviation. The level of miR-142-3p is shown in log scale. N = 3. Error bars present the standard deviation.

Figure 4. Polyacrylamide gel electrophoresis of 3' adaptor adenylation, dephosphorylation and ligation reaction containing the lysis buffer

(A) Phosphorylated 3' adaptor oligonucleotides were subjected to adenylation reaction followed by a dephosphorylation step. Samples subjected to the reaction are indicated by “+” whereas the control samples that were not subjected to the reaction are labeled as “-”. Reaction products were analyzed through denaturing acrylamide gel electrophoresis. A representative gel picture is shown, with the adenylation and dephosphorylation reactions converting >60% 5'-phosphorylated 3' adaptor into an adenylated form. (B) Synthetic miRNAs were ligated to the freshly prepared 3' adaptor (pre-adenylated and dephosphorylated) in reaction buffers containing indicated concentrations of Triton or sodium dodecyl sulfate (SDS). H₂O indicates the control test conducted in distilled water without Triton or SDS. Note that the excess unreacted adaptors still exist after reaction.

Figure 5. Global profiling of miRNAs from whole cell lysate

(A) Heatmap showing the profiling of miRNA expression in NIH3T3 cells, BaF3 cells (WT) and BaF3 cells with miR-142 knockout (KO) cells. Whole cell lysates from 100,000 cells were used directly for miRNA capture and amplification. In this heatmap, representative data are shown from two biological replicates. Each row represents one annotated miRNA. miR-142-5p and miR-142-3p, which are known to be present in BaF3 and ablated in BaF3 KO, are highlighted with black arrows. (B) Scatter plot of normalized miRNA expression levels, which compare BaF3 cells (WT) and BaF3 miR-142 KO cells. Data represent the average from two biological replicates, and data below 4 (log2 scale) are plotted at the level of 4. Each dot represents one annotated miRNA. Again, miR-142-5p and miR-142-3p are highlighted with black arrows. (C) Scatter plot of normalized miRNA expression levels showing the correlation between two biological replicates (from NIH3T3 cells). Each dot represents one annotated miRNA. The correlation coefficient is $R = 0.988$.

Figure 6. Global profiling of miRNAs from low quantities of cell samples
 (A) Scatter plot of miRNAs (normalized expression level), comparing the data from 5000 and 1500 BaF3 cells (WT). Data represents the average from biological duplicates. The correlation coefficient $R = 0.962$. (B) Scatter plot of miRNAs (normalized expression level) comparing the data from 5000 and 500 BaF3 cells (WT). The correlation coefficient is $R = 0.939$. Data represents the average from two biological duplicates. Each dot represents one annotated miRNA. miR-142-3p, miR-let-7b and miR-let-7c are indicated with arrows.