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Protocols

Preparation and processing of dried blood spots for microRNA sequencing

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Abstract

Dried blood spots (DBS) are biological samples commonly collected from newborns and in geographic areas distanced from laboratory settings for the purposes of disease testing and identification. MicroRNAs (miRNAs)—small non-coding RNAs that regulate gene activity at the post-transcriptional level—are emerging as critical markers and mediators of disease, including cancer, infectious diseases, and mental disorders. This protocol describes optimized procedural steps for utilizing DBS as a reliable source of biological material for obtaining peripheral miRNA expression profiles. We outline key practices, such as the method of DBS rehydration that maximizes RNA extraction yield, and the use of degenerate oligonucleotide adapters to mitigate ligase-dependent biases that are associated with small RNA sequencing. The standardization of miRNA readout from DBS offers numerous benefits: cost-effectiveness in sample collection and processing, enhanced reliability and consistency of miRNA profiling, and minimal invasiveness that facilitates repeated testing and retention of participants. The use of DBS-based miRNA sequencing is a promising method to investigate disease mechanisms and to advance personalized medicine.

Keywords: miRNome; biomarkers; circulating markers; minimally-invasive biosampling; high-throughput

Introduction

The strengths of using dried blood spot samples for disease identification

Reliable measurement of molecules is a cornerstone of biological methods aimed at identifying candidate biomarkers with clinical applicability. Blood samples serve as a primary biospecimen in identifying molecular biomarkers of disease [1]. The protocol presented here addresses the need for a streamlined, cost-effective, and minimally burdensome approach for collecting, processing, and measuring molecular markers in serological samples.

Dried blood spots (DBS) are routinely used in newborn screening (e.g. heel prick in infants) and in adults (e.g. finger prick for diabetes monitoring) to identify genetic and metabolic diseases (for more information, see [2, 3]). This globally accepted practice has high analytical sensitivity and specificity—i.e. correctly detecting individuals with a disease and verifying negative result in healthy persons [4]. Although DBS are collected for a singular purpose where sampled blood is used up for immediate analysis, in some instances, residual samples are preserved for secondary use [5]. The application of DBS-based samples in biomolecular marker studies spans diverse fields, from monitoring environmental health and infectious diseases [6, 7] to cancer detection and therapeutic evaluation [8–10]. The minimally invasive nature of DBS sampling facilitates frequent sample collection across a wide range of demographic groups and conditions, offering substantial advantages in disease risk assessment, diagnostics, and monitoring [6, 11].

DBS collection presents a viable alternative to venipuncture. It requires minimal training, does not necessitate a laboratory setting or infrastructure, and is well-suited for individuals sensitive to pain. Molecular profiles obtained via DBS are highly correlated with those obtained from whole blood or plasma samples [12, 13], indicating a broad representation of biological activity. Moreover, this method enhances participant retention and promotes efficient disease monitoring in longitudinal assessments. The analytical reliability of archived DBS samples for up to 20 years [14–17] provides an invaluable opportunity to serve as a snapshot of the past for epidemiological and longitudinal biomarker studies.

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Other biosampling techniques, such as liquid whole blood or plasma, demand professional handling in the pre-analytical stage [13, 18, 19], along with a stabilizing agent (e.g. preservation tubes as PAXgene and EDTA) for long-term storage, prompt centrifugation [20], and significantly larger freezer and transport accommodations than are required for paper cards [21]. Therefore, the collection of DBS specimens can also prevent potential pre-analytical errors. In Canada and the USA, DBS is classified as a non-regulated and exempt material, allowing easy shipping in an envelope [22], and providing access to residual samples from clinical testing for research purposes. It is advisable, however, for researchers to consult the regional and country-specific regulations. Special attention does need to be paid to the size of DBS punches, shipping time, and exposure to high temperature or humidity [23, 24]. Although DBS specimens do not require freezing, opting for temperatures below −20°C maximizes the reliability of gene expression measurements [25].

DBS can provide a valuable insight into both intra- and extracellular components of blood [13]. The matrix of the filter paper binds a variety of biomolecules including cells, protein, antibodies, antigens, DNA, RNA, and various other forms of nucleic acids [26]. As such, DBS is a useful tool for researchers studying complex conditions that affect systemic functions, intracellular markers, microenvironments, or candidate molecules that have organ- and/or cell-specific expression.

MicroRNAs as regulators of gene expression in response to environmental factors

Changes in gene expression can be used as an indicator of the ongoing interplay between environmental factors and biological function at a given time. MicroRNAs (miRNAs) have garnered considerable attention for their role as molecular markers of pathology risk [27–30] and as potential therapeutic agents [31, 32] due to their ability to regulate gene expression on a broad scale. The functional implications of miRNAs range from fine-tuning local intracellular gene activity to modulating entire networks of signaling pathways.

MiRNAs, released from their cell of origin in response to specific signals, are abundant in body tissues and biofluids, and serve as signaling molecules across different cells and organs [33]. Their expression levels can be assessed in peripheral fluids, such as whole blood and saliva, and can be used as a proxy for their expression levels in specific tissues, including the brain [34–36]. MiRNAs are present in cell-free body fluids (e.g. cerebrospinal fluid or plasma), shielded by RNA-binding proteins, or encapsulated within extracellular vesicles [37]. MiRNAs are highly stable and readily detectable in peripheral fluids, making them a valuable asset for potential use as therapeutic agents, with the capacity to reach the brain [21, 38, 39].

MiRNAs' robust resistance to degradation under conditions that would break down most RNAs and widespread influence over the post-transcriptional landscape makes them ideal candidates for biomarker studies across developmental, aging, and disease-related pathways [40, 41]. Researchers have begun to appreciate the stability of the miRNome, as studies have found miRNA levels in DBS samples on par with those in liquid blood [9, 12]. As miRNA processing and degradation are tightly orchestrated [42–44], temporal snapshots of circulating miRNAs can reveal dynamic shifts in cellular processes, thus serving as a chronicle of biological events or interventions. The robust measurement and recovery of miRNAs in DBS samples paves the way for the study of various topics, including fetal programming of metabolic diseases [45] and individual fitness scores [46].

Harnessing the power and advancements of small RNA next-generation sequencing

DBS biospecimens have been analyzed with Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) [47], microarray [48], genotyping [49], genome-wide analysis [50, 51], and next-generation sequencing (NGS) [52]. Methods such as RT-qPCR require controls, have limited throughput, and can be sensitive to user practices and reaction efficiency. In contrast, NGS offers a species-independent method, eliminating reliance on endogenous controls, enabling re-analysis with updated human genome templates, and revealing miRNA variants. NGS surpasses microarray technology by demonstrating superior intra-sample and inter-lab gene expression replicability [53], and overcoming several limitations, such as reliance on predesigned mature miRNAs detection probes, background noise (cross-hybridization), lack of isoform or anti-sense transcript identification, and limited sensitivity for low-abundance transcripts [54–56].

To prepare samples for sequencing and create a sequencing-ready library from short miRNA molecules, adaptors are ligated to the ends of the miRNAs. These adaptors serve the dual purpose of adding primer-binding sites for reverse transcription and enabling subsequent amplification steps. The protocol we outline here addresses a common limitation of most miRNA sequencing studies: the sequence bias from preferential adapter binding affinity at the 5′ and 3′ ends of the input sequences [57–60]. This bias can be mitigated by introducing modified forms of RNA ligases (T4 RNA ligase), random bases on adapter ends and optimizing polyethylene glycol (PEG) concentrations [60–63]. While various approaches are being explored, employing random bases (also known as degenerate bases) at the ligation boundary has emerged as an effective strategy to overcome ligation-based biases [64–68]. MiRNAs have been observed to preferentially and more efficiently ligate to sequences that enable structural interactions [65, 69–71]. The incorporation of random bases accommodates natural sequence variation in miRNAs, reducing the likelihood of self-ligation and facilitating the capture of miRNA diversity. For in-house library preparation, we took the approach of Extracellular RNA Communication Consortium initiative [72] and the Galas et al. protocol (Galas Lab 4N RNA library preparation protocol A—Version 1.0—Pacific Northwest Research Institute, Seattle University, USA) [62, 73], documented to have high reproducibility across labs, to capture sequence diversity, and to consistently deliver reliable results—even in situations of low RNA input, including in extracellular-vesicle derived miRNA samples [74].

Preserving RNA quality (e.g. messenger RNA) in DBS is a shared challenge with postmortem tissue studies, given potential varying degradation rates among samples [75] and volume diminishment in unstable temperature conditions. However, the association between RNA integrity number (RIN) values, indicating the degradation of full-length RNA, and small RNAs is virtually nonexistent [76]. Our protocol confronts the issue of low RNA input by adopting a modified approach that quantifies RNA with linear fluorescence detection (Table 1) for successful library preparation and NGS analysis of miRNAs.

By refining and amalgamating different protocols, here we describe a highly selective sequencing approach for miRNA detection, incorporating advancements in DBS RNA extraction and library preparation techniques suitable for low-input miRNA samples. The extraction (Table 2) and library construction procedures we outline minimize noise, maximize efficient use of

Table 1. The specifications for Nanodrop indicate a range of detection between 2 and 12 000 ng/μl, which is not a suitable quantification method for DBS RNA.

	Nanodrop		RiboGreen
Sample ID	ng/μl	260/280	pg
T43	−1.71	0.71	0.77
E164	−1.65	0.75	0.40
G1	1.7	2.23	0.95
G2	29.2	1.46	2.577864
G3	14.1	1.45	1.498758
G4	11.3	1.45	0.415353
S1	0.9	2.12	0.00072
S2	2.3	2.04	1.21
T1	37.9	1.45	4.690695
T2	0.9	10.32	2.36
T3	17.4	1.47	2.10065
T4	10.2	1.5	1.050205
M1	2.4	3.1	0.82
M2	16.8	1.37	1.118038
M3	1.6	4.55	0.71
M4	11.5	1.51	0.864861
M5	8.5	1.49	0.14068

Fluorescence-based quantification methods, such as the modified method described in this protocol with Quant-iT RiboGreen kit are significantly more sensitive.

Table 2. We tested five modification of RNA extraction protocols to select the procedure that yields the highest concentration of total RNA.

Method	Sample ID	pg/μl
RLT lysis buffer	G	0.95
	S	0.001
	M	0.82
QIAzol/	G	**23.13**
Phenol based	T	**16.43**
	M	**12.28**
RLT buffer + β-mercaptoethanol	S	1.21
	T	2.36
	M	0.71
Tris–EDTA Buffer	G	16.58
	T	15.40
	M	10.64
Overnight with QIAzol	G	7.97
	T	6.83
	M	5.64

Note: Incubating the DBS with QIAzol, followed by sonification and addition of chloroform, as per the miRNeasy protocol, shows consistently high concentrations.
Highlighted in bold are the RNA concentration values for three samples measured with the phenol based method, showing the highest yield.

sequencing resources (Figs 1 and 2), and select for small noncoding RNAs—predominantly composed of miRNAs (Fig. 3). Given the dramatic decrease in NGS costs [77], our approach allows for the assessment of detailed global miRNome profiling at an affordable cost. In addition, freely accessible and user-friendly institutional/web services for small noncoding RNA sequencing analysis are now available to aid in standardizing data processing and analysis, including miRMaster2 [78]; sRNAPipe via Galaxy [79], Genboree [80], and miRDeep2 [81]. The analysis in the protocol, we detail below was guided by the exceRpt pipeline on Genboree server, designed to identify different types of small RNAs (http://genboree.org/java-bin/workbench.jsp).

Our method of sequencing miRNA in DBS provides a straightforward yet robust approach for comprehensive molecular profiling, suitable for hypothesis-driven and exploratory research alike. DBS biosampling circumvents technical challenges in sample processing and storage, enabling the capture of representative miRNA profiles in blood that facilitates comprehensive (e.g. repeated assessments within an individual) and large-scale (e.g. population-level) testing. Since only a single punch of each DBS sample is needed for the miRNA sequencing, the rest of the paper card can be preserved for additional analysis, such as genomic, proteomic, or metabolic profiling, in the same subject.

Specific considerations for implementing DBS MiRNA sequencing protocol

The procedure is structured into the following sections: (i) sample collection and storage; (ii) sample preparation and RNA extraction; (iii) RNA quantification; (iv) library preparation; and (v) RNA sequencing and analysis.

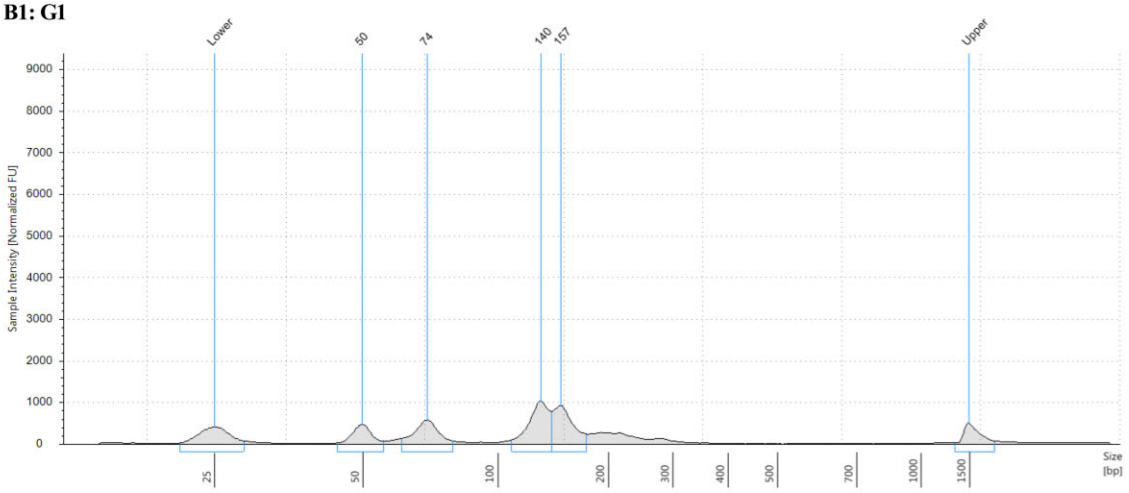
The critical elements of the first section involve collecting blood without smearing, maintaining storage at −20°C or lower, and ensuring controlled humidity conditions. The blood volume procured from finger-pricks relates to lancet penetration depth, with recommended length not exceeding 2.4 mm for individuals 8 years of age and older (for detailed guidelines, refer to WHO Guidelines on Drawing Blood [82]). In the literature, there is variability in the DBS punched disk diameter (ranging from 3 to 9 mm) and the number of spots used in downstream analysis. Based on adequate spot sizes observed in adolescent and young adult participants [83], we opted for a single punch of a 6-mm diameter.

We conducted a comparative analysis of several RNA extraction protocol modifications, based on different approaches described in the literature, and using three DBS samples. As shown in Table 2, we tested: (i) addition of RLT buffer (proprietary name for a lysis buffer in the RNeasy kit) during DBS incubation and agitation [84]; (ii) an adaptation of miRNeasy kit with QIAzol added prior to DBS incubation and agitation, followed by sonication, and subsequent addition of chloroform [85]; (iii) incubation with RLT with β-mercaptoethanol [86]; (iv) incubation with Tris–EDTA (TE) buffer [45]; and (v) overnight incubation with QIAzol [9]. The highest concentrations were observed consistently with the QIAzol agitation and sonication approach, which we adopted in this protocol.

Our protocol approach relies on purifying the miRNA enriched fraction (<200 nucleotides) to remove genomic DNA and large RNAs (this fraction can also be retained). MiRNA enriched samples compared with total RNA fractions within the same subjects show higher diversity in profiled reads (Table 3). Although the sorting of small RNAs is desired, it poses a challenge for the RIN, which relies on ribosomal RNA; thus, making conventional measures such as Nanodrop unsuitable for accurately estimating the input material (Table 1). We used an adaptation of the Quant-iT RiboGreen RNA Assay kit, allowing us to estimate RNA on the picogram scale (Table 2). As an alternative to the RiboGreen assay, it is possible to assess RNA quantity, quality, and the presence of small RNAs with Bioanalyzer (Agilent Technologies), although we should note that the accuracy of the Bioanalyzer for measuring low quantities can be limited, and intercalating dyes, such as RiboGreen, may enhance the reliability and consistency of the results.

Our recommended small RNA and complementary DNA (cDNA) library preparation mitigates the potential biases related to ligase binding and sequence-specific predilection. The cost of reagents is lower compared with commercial options. The limited number of Illumina indices possible in one pool of samples per lane can be overcome with a custom design of the barcodes.

A **B1: G1**



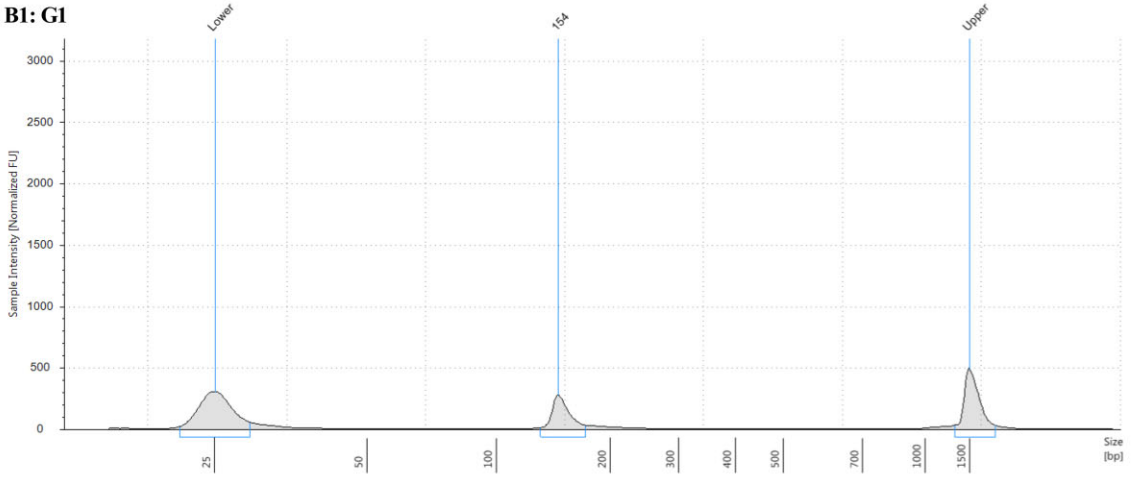
Sample Table

Well
B1

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	% Integrated Area	Observations
25	403	-	-	Lower Marker
50	286	-	14.47	
74	432	-	21.89	
140	653	-	33.06	
157	604	-	30.58	
1500	250	250	-	Upper Marker

B **B1: G1**



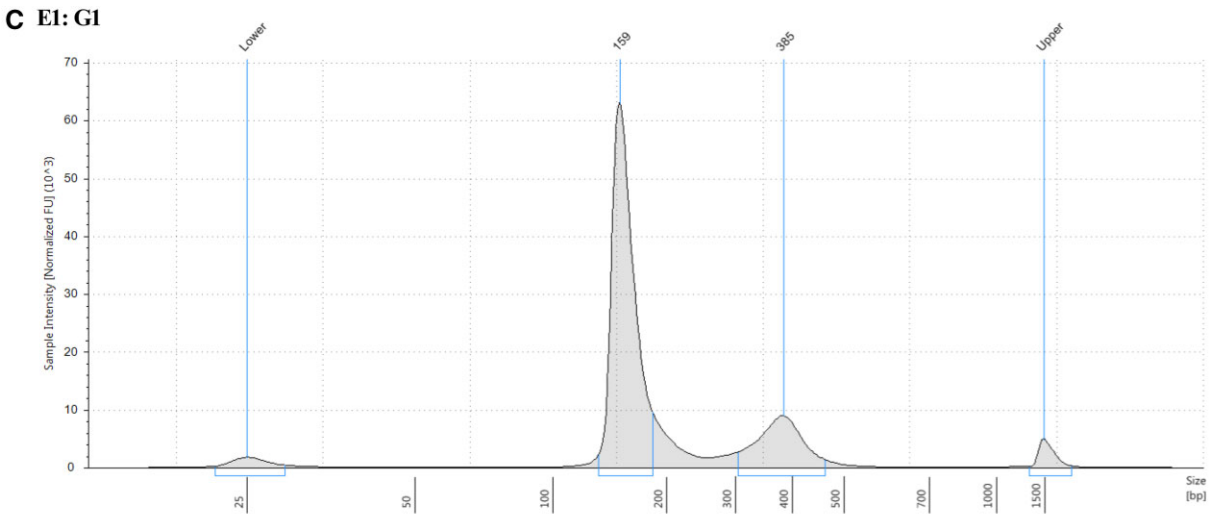
Sample Table

Well
B1

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	% Integrated Area	Observations
25	354	-	-	Lower Marker
154	159	-	100.00	
1500	250	250	-	Upper Marker

Figure 1. Example concentration peaks of a single DBS sample measured with TapeStation across the library preparation steps: (A) after PCR#1, (B) after gel purification with BluePippin, and (C) after PCR#2. The final library amplification step (note the change from picograms to nanograms) plays a crucial role in enhancing the representation of small RNA molecules, ensuring their adequate abundance for downstream sequencing analysis.



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
E1	149	G1	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Peak Comment	Observations
25	5.30	-	326	-		Lower Marker
159	116	-	1130	78.19		
385	32.5	-	130	21.81		
1500	6.50	6.50	6.67	-		Upper Marker

Figure 1. Continued.

This protocol accommodates low sample input preferences, obviates the need for high quality RIN values or internal controls, and incorporates a library preparation that substantially minimizes binding biases. To our knowledge, some limitations remain, including potential preferential PCR amplification of shorter fragments in samples with low RNA input, and the under-amplification of miRNAs possessing complex secondary structures.

Reagents and equipment

Materials

Sample collection and storage

- Mini contact-activated lancets (BD 366594 Microtainer, BD Biosciences)
- 3 mm filter paper cards (Whatman #903, GE Healthcare)
- Double-lock Ziplock bags
- Desiccant packs
- Humidity indicator card—30%, 40%, 50% (e.g. ULINE S-1547)
- Alcohol pads (70%)
- Band-aids and gauze

Sample preparation

- Single hole punching pliers with a 6-mm diameter punch head
- Particle-free and gentle wiping material—e.g. Kimwipes (e.g. Fisher scientific Cat. No. 06-666)
- RNAase free, labeled Eppendorf tubes 1.5–2 ml (e.g. Invitrogen Fisher scientific Cat. No. AM12425)
- Holder for Eppendorf tubes
- Forceps
- RNaseZap (e.g. Fisher scientific Cat. No. AM9780), diethylpyrocarbonate-treated water (DEPC H_2O) for cleaning
- PCR tubes 0.2 ml 8-tube strips (Eppendorf Cat. No. 0030124286)
- Dry ice

Sample RNA quantification

- Microplate with optical transparency, such as the Greiner Bio-One FLUOTRAC 96-well non-treated microplates (Fisher scientific Cat. No. 07000721 or equivalent)
- Pipet-Lite Multi Pipette L8-200XLS+ (Rainin, Cat. No. 17013805)

Absolute quantification of libraries

- PCR tubes 0.2 ml 8-tube strips (Eppendorf Cat. no. 0030124286)
- 96-well PCR plate, e.g. Eppendorf, Cat. No. 30129504
- Adhesive PCR Plate Seal, e.g. Bio-Rad, Cat. No. MSB1001

Reagents

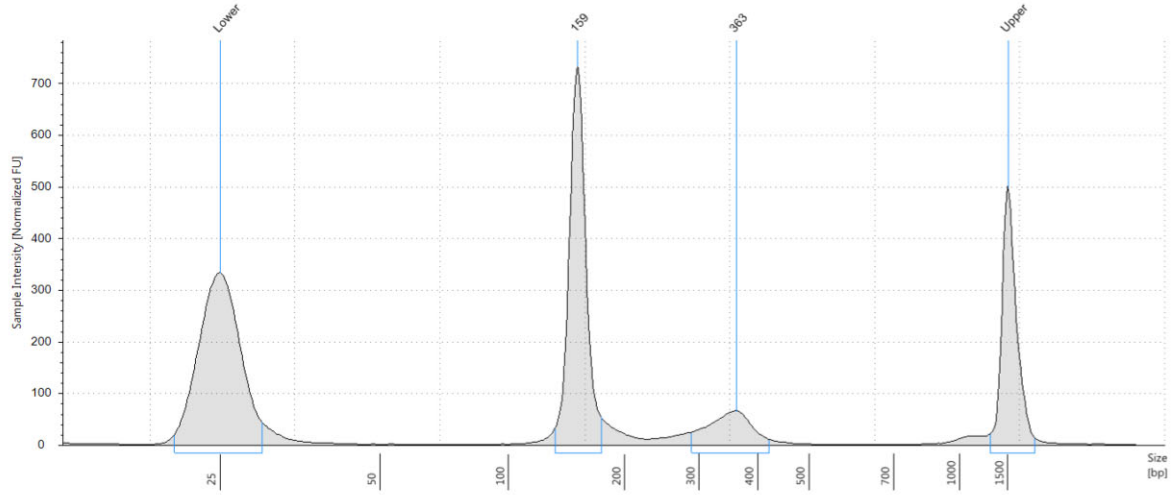
RNA extraction

- miRNeasy Micro kit (Qiagen, Cat. No. 217084)
- Ethanol 100% (Sigma, Cat. No. 459836-500ML)
- RNase-free chloroform

Sample RNA quantification

- Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen Cat. No. R11490)

A **A2: G1 beads**



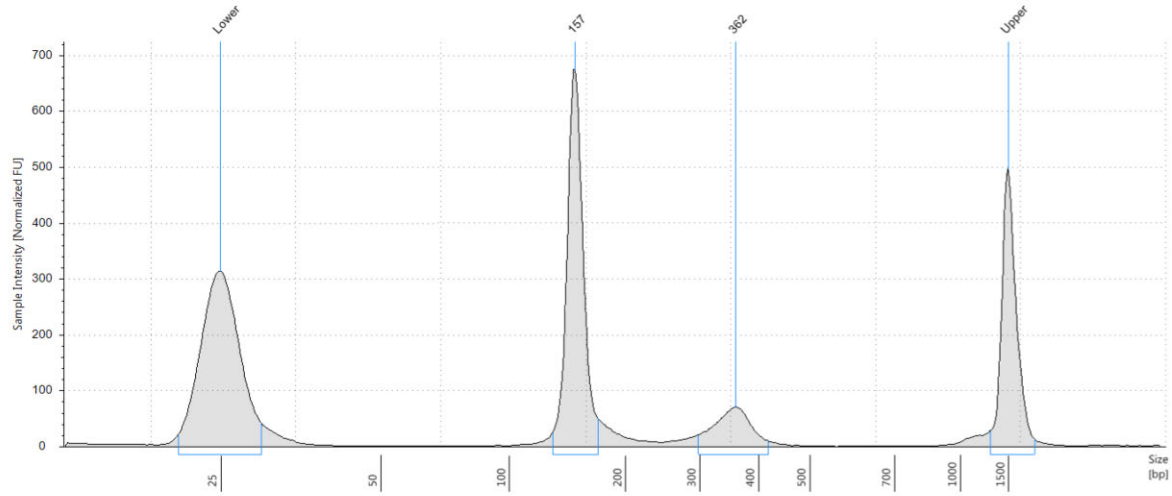
Sample Table

Well
A2

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	% Integrated Area	Observations
25	474	-	-	Lower Marker
159	415	-	81.29	
363	95.5	-	18.71	
1500	250	250	-	Upper Marker

B **F1: G1 beads twice**



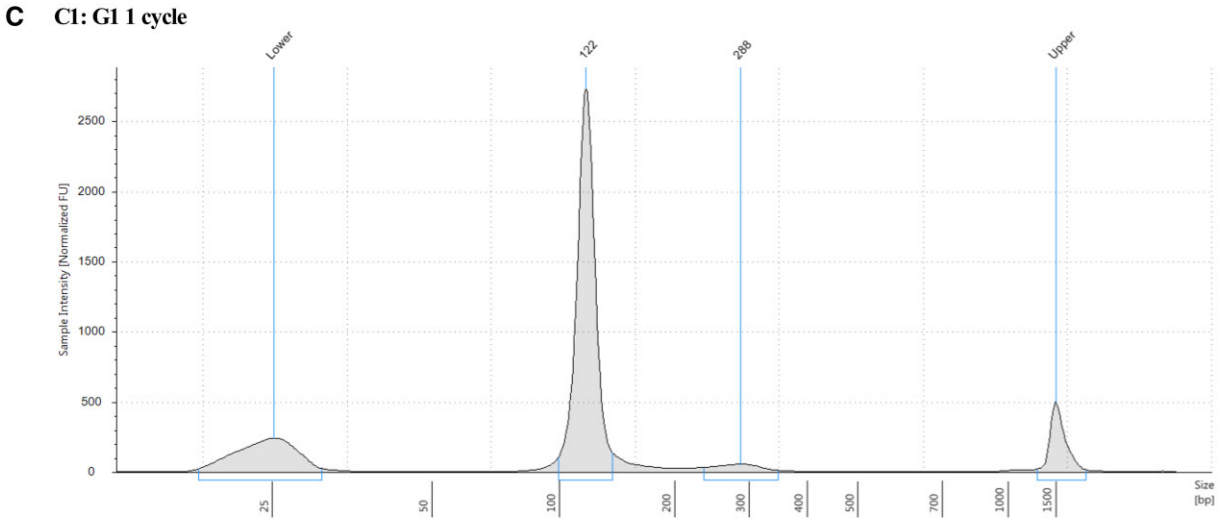
Sample Table

Well
F1

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	% Integrated Area	Observations
25	422	-	-	Lower Marker
157	375	-	81.18	
362	86.9	-	18.82	
1500	250	250	-	Upper Marker

Figure 2. Comparison of AMPure beads purification versus reconditioning PCR to eliminate the PCR "bubble" products, of a library diluted to 2 ng/µl. (A) The sample (size 159 bp) concentration 415 pg/µl and bubble product (size 363 bp) at 95.5 pg/µl after a single and (B) a second round of purification with AMPure beads, with final sample (size 157 bp) concentration of 375 pg/µl and bubble product (362 bp) 86.9 pg/µl. The beads purification slightly reduced the size of the PCR bubble at a cost of losing sample concentration, and a repeated purification did not improve the sample to bubble ratio. An alternative method is to expose the library to a single cycle of PCR#2 (C) which substantially reduces the large PCR bubble product (size 288 bp 85.1 pg/µl) without losing the sample (size 122 bp 1.63 ng/µl).



Sample Table

Well
C1

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	% Integrated Area	Observations
25	490	-	-	Lower Marker
122	1630	-	95.05	
288	85.1	-	4.95	
1500	250	250	-	Upper Marker

Figure 2. Continued

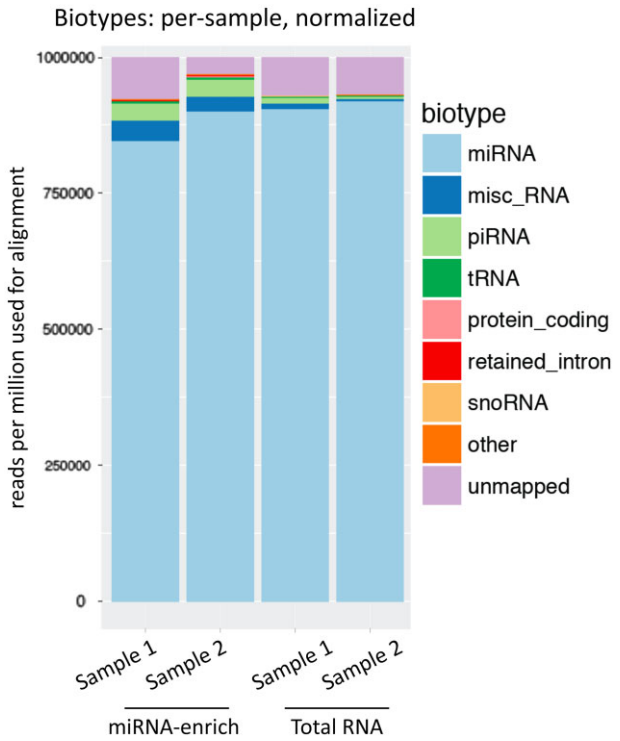


Figure 3. Reads by biotype reveal substantially greater proportion of miRNAs across all samples (either enriched for small RNA during extraction versus total RNA). Figure obtained from ExceRpt pipeline results.

Table 3. Biased representation of a single miRNA in total RNA extraction.

	miR-enriched extraction		Total RNA extraction	
miRNA ID	**Sample 1**	**Sample 2**	**Sample 1**	**Sample 2**
miR-451a	24.34%	10.98%	52.61%	44.46%
miR-486-5p	13.19%	27.47%	3.99%	8.54%
miR-92a-3p	8.62%	12.46%	3.25%	4.17%
let-7a-5p	5.37%	4.15%	3.44%	3.41%
hsa-miR-16-5p	3.28%	2.48%	3.01%	3.40%

Notes: Shown top five miRNAs, with each value indicating percent of expression out of all mapped miRNA reads. miRNA enriched extraction shows higher diversity of reads profiled.

Library preparation

Oligonucleotide sequences (e.g. custom order from Integrated DNA Technologies (IDT))

- 5′-adapter (desalted)—5′-rGrUrUrCrArGrArGrUrUrCrUrArCr ArGrUrCrCrGrArCrGrArUrCr(N : 25252525)r(N)r(N)r(N)
- 3′-adapter (HPLC purification)—5′/5rApp/(N: 25252525)(N)(N) (N)TGGAATTCTCGGGTGCCAAGG/3ddC/
- RT primer (desalted)—5′-GCCTTGGCACCCGAGAATTCCA
- RP1 PCR primer (HPLC Purification)—same as Illumina RP1 PCR primer
- Indexed Illumina PCR primers, e.g. RPI1–RPI48 (HPLC Purification)
- Universal PCR primer F (desalted)—5′-AATGATACGGC GACCACCGAG

- Universal PCR primer R (desalted)—5′-CAAGCAGAAGACGGCATACGA

Reagents for 3′-ligation

- T4 RNA ligase 2 truncated KQ (NEB M0373)
- 10× T4 RNA ligase reaction buffer (included with T4 RNA ligase)
- 50% PEG 8000 (included with T4 RNA ligase)
- RNAseOut RNAse inhibitor (Invitrogen 10777-019)
- Strip tubes (Axygen PCR-0208-CP-C or equivalent)

Reagents for adapter depletion

- *Escherichia coli* single-stranded DNA binding protein (SSB) (Promega M3011)
- 5′ deadenylase (NEB M0331)
- RecJf (NEB M0264)

Reagents for 5′-ligation

- 10 mM ATP (NEB P0756)
- T4 RNA ligase 1 (NEB M0204)

Reagents for reverse transcription

- Strip tubes (Axygen PCR-0208-CP-C or equivalent)
- Superscript III (Invitrogen 18080-044)
- 5× First strand buffer (included with Superscript III)
- 0.1 M dithiothreitol (DTT) (included with Superscript III)
- 25 mM deoxynucleotide triphosphates (dNTP) mix (Thermo Scientific R1121)
- RNAseOut RNAse inhibitor (Invitrogen 10777-019)

Reagents for PCR amplification #1

- NEBNext Ultra II Q5 PCR master mix (NEB M0544) or other high fidelity PCR master mix
- DNA clean and concentrator—5 columns (Zymo D4004)

Reagents for gel purification

- 3% agarose cassettes for BluePippin system (Sage Science BDF3010)

Reagents for PCR amplification #2

- KAPA 2× real-time PCR master mix (KAPA KK2701) or other high fidelity PCR master mix
- DNA Clean and Concentrator 5 columns (Zymo D4004)

Reagents for library validation

- TapeStation high sensitivity D1000 sample buffer (Agilent, Cat. No. 5067-5603)
- TapeStation high sensitivity D1000 ScreenTape (Agilent, Cat. No. 5067-5584)

Absolute quantification of libraries

- KAPA SYBR FAST qPCR Master Mix (Roche KK4600)
- PhiX Control v3 (Illumina FC-110-3001)
- Illumina P5 primer (IDT custom: 250 nmole DNA Oligo 5′-AATGATACGGCGACCACCGA)
- Illumina P7 primer (IDT custom: 250 nmole DNA Oligo 5′-CAAGCAGAAGACGGCATACGA)

Equipment

Sample storage

- Freezer (−20°C short term, −80°C long term)

RNA extraction

- Sonicator
- Agitator with regulated temperature setting
- Centrifuge

Sample RNA quantification

- A fluorescence plate reader, e.g. Tecan Spark

Library preparation

- Vacuum concentrator, e.g. Savant SpeedVac Concentrator (Thermo Fisher Scientific)
- Thermocycler
- TapeStation system 2200 (Agilent) or equivalent equipment
- BluePippin System (BLU0001; required for BluePippin gel cassettes for the automatic size selection step)
- qPCR machine, e.g. QuantStudio™ 6 Flex System

Absolute quantification of libraries

- qPCR machine, e.g. QuantStudio™ 6 Flex System

Procedure

Sample collection and storage

1. Request that the participant warm their non-dominant hand under comfortably hot water for 2 min.
2. Label the collection paper card (Whatman).
3. Dry participant's hands with paper towel and immediately clean the middle/ring finger of the non-dominant hand with isopropyl alcohol wipes. Allow the finger to air dry for 30 s.
4. Use the lancet (BD Microtainer) to prick the side of the selected finger, avoiding the fingerpad.
5. Gently apply pressure to the sides of the finger to increase blood flow and formation of a blood droplet. If necessary, ask participant to stand and relax their arm while you massage their hand to further stimulate blood flow.
6. Capture the initial blood drop with a gauze pad and dispose of it in a biohazard bag.
7. Position the participant's hand above the paper card, if necessary, applying gentle pressure, to produce another drop of blood. Touch the blood droplet with the filter paper, absorbing the blood without the paper contacting the skin. IMPORTANT: Refrain from smearing the blood onto the collection paper. Avoid placing additional blood on a previously spotted area, even if the initial spot appears small. Overlaying blood drops can concentrate the sample and compromise the uniform diffusion properties of the paper.
8. Saturate each circle indicated on the paper card with a drop of blood (one drop per circle).
9. If necessary, repeat for all circles using the same finger prick.
10. Apply a bandage to the pricked finger.
11. Record the sample collection time and any additional notes on the data collection form.
12. Store the labeled card in a temporary storage box made of non-absorbent material. This box will protect the cards against direct sunlight and heat. Allow the card to dry fully at room temperature under low humidity conditions for a minimum of 3 h, or leave it overnight, maintaining consistent drying times across all samples. Do not stack the cards during storage.

13. Once dry, the card should be placed in a resealable plastic bag with a desiccant sachet. Store the bag at a consistent temperature of −20°C or colder.

Sample preparation (spot punching)

14. Note that the size of punched circle should be consistent across samples, thus it is crucial to use the same hole-puncher for all specimens. We suggest punchers that produce a 6-mm diameter spot and one spot per tube for downstream processing. It is essential to clean the punching pliers between each sample with ethanol and to allow them to dry thoroughly.
15. Maintain a clean working environment by disinfecting the counter with RNAaseZap (Thermo Fisher) + DEPC H_2O. If the samples were stored at freezing temperatures, place a few Ziplock bags containing samples (~2 to begin with, progress to 4, max 6 at a time) atop dry ice—CAUTION: use proper ventilation and take care to avoid freeze burns.
16. Sterilize the forceps that will handle the paper cards and hole-puncher tool with ethanol, ensuring they are fully dry before use. Label the tubes with the sample ID on both the top and the side.
17. When picking up the samples, use caution that no dry-ice precipitation forms on the Ziplock bag, so it doesn't get inside or onto the card. If necessary, wipe the Ziplock bag with a Kimwipe before opening. Grab the sample card by the edge with forceps. Place the puncher over the DBS, while holding the Eppendorf tube (Invitrogen) with another hand—CAUTION to punch the center of the DBS—1 punch per sample per 2 ml tube, not the edge.
18. After the punching process, place the card back with care, slowly sealing the Ziplock bag while gently pressing out any excess of air—to avoid the possibility of condensation from temperature fluctuations. Immediately transfer the bag to a container with dry ice. Close the tube and place on dry ice.
19. Clean the hole-puncher and the forceps (if used) with ethanol pads and wipe them dry with Kimwipes (Fisher Scientific) after each sample.
20. Repeat this process for the remaining samples.

RNA extraction

Note: Due to inconsistency in the literature in regard to RNA extraction procedure, we tested several modifications, including [9, 84–86] (Table 2). The optimized protocol that yields the highest RNA concentration and miRNA proportion (Table 2; Fig. 3) is detailed below.

21. Place the 2 ml tube with DBS on dry-ice until the addition of 1 ml of QIAzol lysis reagent (included in miRNeasy kit; Qiagen).
22. Agitate the tubes at 450 rpm for 15 min at 37°C.
23. Place the tubes in Sonicator at room temperature (15–25°C) for 15 min.
24. Agitate the tubes once again, 450 rpm for 15 min at 37°C.
25. Add 250 µl of chloroform and vortex the tube prior to incubation at room temperature for 5 min.
26. Centrifuge the tube at 12 000 × *g* for 15 min.
27. Remove the tube carefully from the centrifuge, ensuring the integrity of the different phases within the tube is maintained. Pipette only the upper aqueous phase and transfer to a new 2 ml tube. If carryover occurs, repeat from *step 26*, centrifuging for at least 5 min.
28. Add 100% 800 µL of ethanol and mix.

Purify the homogenate for miRNA-enriched fractions, following Appendix A of the manufacturer's miRNeasy handbook (Qiagen):

29. Pipet the sample into RNeasy MinElute spin column within a 2-ml collection tube. Gently close the tube, centrifuge at 12 000 × *g* for 30 s at room temperature. Pipet the flow-through with the miRNA fraction into a new 2-ml tube. Discard the RNeasy MinElute spin column.
30. Add 500 µl of 100% ethanol (×0.65 volumes) to the flow-through and vortex to mix thoroughly.
31. Pipet 700 µl of the sample into a new RNeasy MinElute spin column placed in a 2-ml collection tube. Centrifuge for 30 s at ≥8000 × *g* at room temperature. Discard the flow-through. Repeat until entire volume of the sample has been processed.
32. Add 700 µl of Buffer RWT to the spin column, close the lid, and centrifuge for 30 s at ≥8000 × *g* at room temperature. Discard the flow-through.
33. Add 500 µl of Buffer RPE to the spin column, close the lid, and centrifuge for 30 s at ≥8000 × *g* at room temperature. Discard the flow-through.
34. Add 500 µl of 80% ethanol to the spin column, close the lid, and centrifuge for 2 min at ≥8000 × *g* at room temperature. Place the spin column into a new 2 ml collection tube, discarding the one with the flow-through to avoid ethanol carryover.
35. Centrifuge the spin column in the collection tube with open lid for 5 min at ≥8000 × *g* at room temperature, positioning the caps into an empty preceding space of the rotor to minimize the risk of caps being torn off.
36. Elute the extracted RNA with 20 µl of RNase-free water into a 1.5-ml collection tube, centrifuging for 1 min at ≥8000 × *g* at room temperature.
    If not proceeding to sample quantification immediately, secure the labeled tube and keep in −80°C for long-term storage.

Sample quantification

Note: Nanodrop can be used for RNA quantification on these samples, however the 260/280 ratios are likely to be inadequate or abnormal due to enrichment of shorter RNA fragments and low concentrations in the majority of samples (Table 1). To this end, we modified the RiboGreen protocol (Invitrogen) which identifies RNA on a scale ranging from 1 ng to 100 pg.

37. Prepare 2 µg/ml RNA standard and 200-fold dilution of RiboGreen (10 µl per standard) with the final concentrations being 1 ng/µl, 500 pg/µl, 250 pg/µl, 100 pg/µl, and blank (1× TE buffer only), with total volumes of 20 µl per well, as such:

1× TE (µl)	2 µg/ml RNA standard	200-Fold Dilution RiboGreen	Final RNA concentration ng/µl
0	10	10	1 ng/µl
5	5	10	500 pg/µl
7.5	2.5	10	250 pg/µl
9	1	10	100 pg/µl
10	0	10	Blank

38. Mix 1 µl of each stock RNA (sample) with 9 µl TE buffer in separate tubes.
39. To a microplate with optical transparency, pipette the RNA standard ladder in duplicates (10 µl per well) and the diluted samples, keeping track of the sample-well placement.
40. Top up the sample wells with the 10 µl of the RiboGreen dilution, gently pipette the solution up and down, and incubate the plate at room temp for 5 min covered with foil.
41. Using a fluorescence plate reader machine (e.g. Tecan Spark), select the appropriate choice for the plate setting, with shaking for 5 s, fluorescence intensity: excitation 480, emission 520.
42. Perform the calculations of the concentrations. The average blank value will be subtracted from all the averaged RNA standard values and the samples, and these sample values multiplied × 10 to return ng/µl concentrations.

Library preparation and pooling

Note: We adopted the Galas protocol "Library Preparation for small RNA sequencing using 4 N adapters" to prepare the samples for sequencing, incorporating minor modifications:

43. Bring the samples to equal concentrations as per calculations from *step 42*.
44. Halve the stated concentrations of the adapters, RT primer, dNTP mix, and Universal primer cocktail compared with the indicated concentration (due to low RNA input and sufficient efficiency). Illumina forward primer and RPI1 through 48 reverse index primers can be used at 10 mM concentrations.
45. Add 3 µl of 50% PEG 8000 (included as such with T4 RNA ligase; NEB) to each strip tube per sample, slowly pipetting the volume of the viscous solution, which can be dehydrated in a vacuum centrifuge/concentrator (set for 37°C, 1–2 h) to create a highly concentrated pellet.
46. For 3′ ligation, in each tube with the PEG pellet, mix 7 µl of stock RNA with 1 µl adenylated 3′ adapter.
    Heat the tube to 70°C for 2 min in a thermocycler, then snap cool on ice.
    To each tube of denatured RNA and adapter add:

    1 µl of 10× T4 RNA ligase reaction buffer
    1 µl RNAseOut RNAse inhibitor
    1 µl T4 RNA ligase 2 truncated KQ

    Incubate at 25°C in a thermocycler for 2 h.
    Add 1 µg *E. coli* SSB (diluted in 1× ligase buffer; Promega) and incubate at 25°C for 10 min.
    Add 1 µl 5′-deadenylase (NEB) and incubate at 30°C for 1 h.
    Add 1 µl of RecJf (NEB) and incubate at 37°C for 1 h.
47. In separate tubes, add 1 µl of 5′ adapter (25 µM) per ligation and denature at 70°C for 2 min, then snap cool on ice.
    Per tube of denatured 5′-adapter, add:

    1 µl 10 mM ATP (NEB).
    1 µl T4 RNA ligase 1 (NEB).

    Add the 3 µl mix (5′-adapter, ATP, and ligase) to the completed 3′-ligation from *step 46*. The total volume will make up 18 µl, pipette up and down to mix.
    Incubate in a thermocycler at 25°C for 1 h.
48. To begin the reverse transcription, to a new strip tube per sample add 1 µl of RT primer (5 µM) and 6 µl of the ligated RNA, from *step 47*. Leftover RNA to be stored at −70°C or below.
    Incubate at 70°C for 2 min, then snap cool on ice.
    Create a master mix of:

    1 µl Superscript III (Invitrogen)
    2 µl 5× First strand buffer (included with Superscript III)
    0.5 µl 12.5 mM dNTP mix (included with Superscript III)
    1 µl DTT (included with Superscript III)
    1 µl RNAseOut (Invitrogen)
    5.5 µl Total

    Add the 5.5 µl of the master mix to each tube of the 7 µl denatured RNA and primer.
    Incubate at 55°C for 1 h, then at 70°C for 15 min.
49. For the first PCR amplification, create a master mix of:

    25 µl PCR master mix (NEBNext Ultra II Q5 or equivalent)
    2 µl Illumina RP1
    8 µl RNAse free water
    35 µl Total

    Add to the tubes with cDNA from *step 48*.
    Add 2 µl Illumina index primer (RPI 1–48, up to 48 samples to be indexed and potentially pooled; important to keep track of index–sample combination) .
    Amplify for 10 cycles:

98°C	30 s	1×
98°C 60°C 65°C	10 s 30 s 35 s	10×
65°C	2 min	1×

50. Purify and concentrate the PCR product with DNA Clean and Concentrator columns (Zymo) to elute to the final volume of 11 µl. As per the Zymo DNA Clean & Concentrator—5 kit instructions, up to 5 µg of total DNA per column into as little as 6 µl can be processed by performing the steps below:

    If using a new kit, ensure to add the indicated amount of 100% ethanol to the DNA Wash Buffer (e.g. for 25 ml DNA Wash Buffer, add 96 ml 100% ethanol).

    a) In a 1.5-ml microcentrifuge tube, add five times the volume of DNA binding buffer to each volume of DNA sample. Based on the rounded volume of the PCR product from *step 49* add 250 µl. Mix briefly by vortexing.
    b) Transfer the mixture to a provided Zymo-Spin Column in a Collection Tube.
    c) Centrifuge for 30 s. Discard the flow-through.
    d) Add 200 µl DNA Wash Buffer to the column, then centrifuge for 30 s. Repeat this wash step.
    e) Add 11 µl DNA Elution Buffer (10 mM Tris–HCl, pH 8.5, 0.1 mM EDTA) or water (pH > 6) directly to the column matrix (not the walls of the column) and incubate at room temperature for 1 min. Transfer the column to a 1.5-ml microcentrifuge tube and centrifuge for 30 s to elute the DNA.
51. Quantify the PCR product with TapeStation (Agilent) HighSensitivity D1000 tapes (detection range 35–1000 bp) with 2 µl of the DNA sample and 2 µl of the HS D1000 Sample Buffer. Cap firmly and vortex thoroughly for at least 1 min. Spin down prior to loading the tube strip into the TapeStation machine.
52. Using BluePippin (Sage Science) for the single gel purification step, with a target of 138 bp size (Fig. 1), using the entire

volume of the samples collected from *step* 50 (~9 µl) bring the volume up to 30 µl with 1× TE as per the manufacturer's protocol, then add 10 µl (room temp) of the supplied marker/internal standard mix.

At the end of the run collect the samples from the elution well.

53. Purify and concentrate the eluted sample as in *step* 50.
54. Quantify the purified samples with the TapeStation system.
55. For the second PCR amplification, create the following master mix:
25 µl KAPA 2× real-time master mix (KAPA)
2.5 µl Universal primer cocktail (10 µM of each, forward and reverse primers)
27.5 µl Total

Bring up the cDNA volume to 22.5 µl with nuclease-free water, and add master mix.

56. Run for six cycles, which creates sufficient amplification and minimizes "bubble" product from being created (see Troubleshooting section for more details; Fig. 2)

98°C	45 s	1×
98°C 60°C 72°C	15 s 30 s 20 s	6×
72°C	2 min	1×

57. Purify and concentrate the PCR product with DNA Clean and Concentrator columns, as in *step* 50. Elute to 20 µl.
58. Quantify the PCR product with the TapeStation HighSensitivity D1000 tapes (quantitative range 10 pg/µl–1 ng) with 2 µl of sample or with 1 µl of sample D100 ScreenTape (quantitative range 0.1 ng/µl–50 ng/µl).

Singular peaks with size (bp) ranging from 140 to 160 are expected and the second gel purification is not needed (Fig. 2).

To achieve precise absolute quantification of libraries and to ensure an accurate pooling of samples, we recommend performing a qPCR test. The pooling process should take into consideration variations in samples, including balancing factors such as sex, age, and condition in each group. Here, we utilized PhiX as the control template/DNA Standard. PhiX, an adapter-ligated library, is commonly used as a control in Illumina sequencing runs. It is particularly useful for ensuring the quality of sequences in runs involving libraries with low diversity.

59. Dilute libraries with either TE buffer (Tris and EDTA) or distilled water, based on molarity estimated with TapeStation (e.g. range between 1 and 10 ng or 1 and 10 nM), so the diluted concentrations fall within the dynamic range of the standard curve (detailed example below).
60. Prepare a set of fresh serial dilutions of PhiX (10 nM; Illumina) in a 0.2-ml eight-tube strip, following the example provided, with a range of 0.1–0.0015625 nM:

PhiX	Concentration
1	0.1 nM
2	0.05 nM
3	0.025 nM
4	0.0125 nM
5	0.00625 nM
6	0.003125 nM
7	0.0015625 nM
Negative control	0 nM

61. Prepare sufficient reaction mix for the required number of reactions with a total volume of 10 µl, assayed in triplicate:

5 µl KAPA-2X SYBR FAST Universal qPCR Master Mix (2×)
4 µl of each library
OR 4 µl PhiX dilution
1 µl Primer premix (Illumina P5&P7 at 10 µM each)

62. Seal the plate using adhesive seal and centrifuge the plate to 250 × *g* for 1 min
63. Run qPCR, selecting absolute quantification option and the following parameters:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 min	1×
Denaturation Annealing/Extension	95°C 60°C	10 s 35 s	40×
Melt curve analysis		65–95°C	

64. Standard curve is generated based on average quantification cycle (Cq) scores of the PhiX dilutions against their known concentrations, removing replicate outliers, e.g. Cq replicates >0.5 from other, or based on statistical outlier detection. This curve enables estimation of sample concentrations based on their Cq values, allowing determination of the corresponding sample concentration by comparing its Cq value to the standard curve.

Sequencing and analysis

Post-preparation, the samples can be submitted to a service company equipped with appropriate sequencing machines (e.g. Illumina NovaSeq 6000 system). Refer to their specific sample volume and concentration submission requirements, and account for some volume to be used for quality control. Our suggested coverage per sample is 20 M reads.

For analysis, we recommend the exceRpt pipeline (Fig. 3; Supplementary Figures 1), which can be downloaded directly (depending on memory capacity) or accessed via Genboree portal. Maintaining a record of analysis package versions is crucial because the existing Genboree packages, such as exceRpt and DESeq2, may not be the latest versions available. The use of the 4 N adapters needs to be specified in the analysis pipeline or manually removed via cutadapt package. We highly recommend that the maximum allowed mismatched bases in the aligned portion of the read should be set to zero (the default is 1), as miRNAs

are short, and allowing for less stringent alignment can change the results.

Troubleshooting

- Library preparation final PCR results in double peak: In cases in which the sample concentrations are high to begin with (>100 ng post PCR#2), the samples are likely to show a "bubble" product, approximately double the band of the peak concentration (Fig. 2). Although this product will not interfere with sequencing, an efficient way to reduce or remove it is to run an additional PCR cycle on all libraries to eliminate it. All samples would need to undergo an additional single PCR cycle:

98°C	45 s	1×
98°C 60°C 72°C	15 s 30 s 20 s	1×
72°C	2 min	1×

- Few to no sequences mapped to the genome: Using an Illumina MiSeq machine with a 50-bp protocol, the automated exceRpt trimming of the sequences was sufficient. However, sequencing with NovaSeq6000 S1100bp leads to the issue of inadequate trimming and few sequences mapped to the genome. To this end, manually removing the 4N adapters and adjusting the sequence length (via command such as: –cut -50 -a NNNNTGGAATTCTCGGGTGCCAAGG -o) prior to file submission to a pipeline, as specified in the previous section, will successfully prepare the reads for mapping to genome.

Time taken

The following section provides estimated time frames for the different phases of the protocol that may vary depending on the number of participants recruited and the equipment used:

i) Sample collection—collecting blood generally requires less than 30 min per participant, with drying times for the cards ranging from 3 h to overnight.
ii) Sample preparation and RNA extraction—ideally conducted in batches of 12–16 (depending on the centrifuge model and available space), DBS punching and extraction should be executed sequentially to minimize the freeze-thaw cycles. Around 32 samples can be comfortably processed within 8 h, if run manually/without equipment that automates and speeds up the process.
iii) RNA quantification—a single plate, which has 96 wells for about 86 samples would take approximately 3 h.
iv) Library preparation—The stages up to reverse transcription, including preparation, 3′ ligation, and 5′ ligation are expected to take 6–8 h, accommodating around 30 samples. Reverse transcription, PCR amplification, and PCR product purification can comfortably be done within 8 h for the same number of samples. Gel purification speed is limited due to the number of samples or libraries that can be processed at a time using the BluePippin cassette, and expected time from sample preparation to collection per plate is about 2 h. A TapeStation measurement should take about an hour. PCR amplification #2 and PCR product purification can comfortably be done within 4 h for about 30 samples.
v) Library quantification—setting up the dilutions and preparing reagents and samples may take approximately 2 h. The qPCR run and subsequent analysis each take less than an hour.

Anticipated results

- Based on the absence of contamination indicated by the humidity index, placed inside the Ziplock bags with the DBS sample cards and in the container holding all Ziplock bags, it is anticipated that transport, storage, and use of the DBS cards for the punching procedure will not contribute to variability in the outcomes.
- Low RNA concentrations are to be expected for miRNA enriched fractions, as described (Tables 1 and 2). The commercial kits for constructing a library specify recommended RNA volume, ranging from 1 to 200 ng. A study on DBS derived miRNA sequencing describes 500 pg input to produce sufficient library [87]. The protocol described here has been tested on 50 pg (with the smallest RNA concentration from a single DBS being 3.5 pg/μl) and produces superior mapping efficiency—MiSeq at the depth of 38M for 4 samples pool, 84%–90% of reads mapped to human genome (Supplementary Figures).
- Library quality shows high percent of reads used for alignment and reads mapped to genome, with minimal failed quality or contamination (Supplementary Figures).
- miRNA reads compose the highest concentrations compared to other types of RNA (Fig. 3).
- Drawing from our substantial experience in analyzing DBS samples from pediatric and adolescent cohorts, including individuals with psychiatric conditions and healthy counterparts, we expect that the fold changes in differentially expressed miRNAs will be relatively modest compared with what is typically observed in gene expression studies.

Limitations

Certain limitations associated with the DBS-sourced miRNA sequencing approach should be recognized, particularly those relating to pre-analytical factors. Although research indicates that technical variability is minor compared with biological factors, it is critical to ensure proper drying of the blood on the card, and to maintain controlled humidity throughout the process using desiccant packets and humidity index cards [88]. These precautions help mitigate potential variations that may arise from factors such as the number of freeze-thaw cycles, multispotted versus a single punch, or the location within the blood spot [89].

Individual variations in hematocrit can contribute to variability in the proportion of red blood cells in DBS samples [17, 90–92]. Diluting the samples to the lowest RNA concentration after extraction helps account for and mitigate hematocrit-related variations in RNA yield. Comparing liquid blood (and its fractions such as plasma) and DBS sample miRNA expression should be done with caution, as hemolysis can introduce bias to miRNA levels [93–97] and to analytes overrepresented in erythrocytes.

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Authors' contributions

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Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

Conflict of interest statement

None declared.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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