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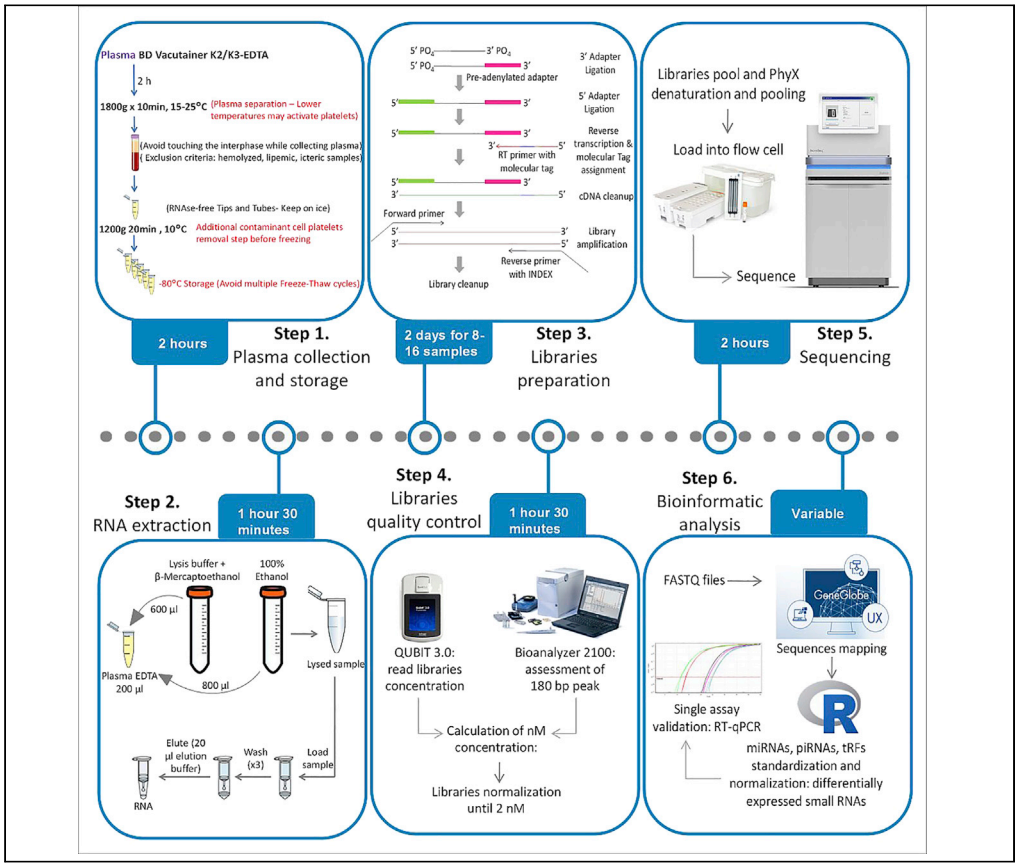
Protocol to analyze circulating small non-coding RNAs by high-throughput RNA sequencing from human plasma samples



The identification and validation of circulating small non-coding RNA (sncRNA) as biomarkers for disease diagnosis, staging, and response to novel therapies is still a compelling challenge. Pre-analytical variables, such as storage temperature or blood hemolysis, and different analytical approaches affect sncRNA stability, detection, and expression, resulting in discrepancies among studies. Here, we report a systematic standardized protocol to reproducibly analyze circulating sncRNAs, employing high-throughput sncRNA sequencing and RT-qPCR validation, from 200 µL of human plasma samples.

Giuseppina E. Grieco, Guido Sebastiani, Daniela Fignani, ..., Chantal Mathieu, Conny Gysemans, Francesco Dotta

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Highlights

Circulating sncRNA sequencing profile from low input human plasma samples

Systematic quality checks on input samples and on cDNA library generation

Identification of sncRNA as potential disease and therapeutic biomarkers

Single-assay validation of candidate sncRNAs

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Protocol to analyze circulating small non-coding RNAs by high-throughput RNA sequencing from human plasma samples

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SUMMARY

The identification and validation of circulating small non-coding RNA (sncRNA) as biomarkers for disease diagnosis, staging, and response to novel therapies is still a compelling challenge. Pre-analytical variables, such as storage temperature or blood hemolysis, and different analytical approaches affect sncRNA stability, detection, and expression, resulting in discrepancies among studies. Here, we report a systematic standardized protocol to reproducibly analyze circulating sncRNAs, employing high-throughput sncRNA sequencing and qRT-PCR validation, from 200 μL of human plasma samples.

For details on the use and execution of this protocol, please refer to Ventriglia et al. (2020), Sebastiani et al. (2017), and Dotta et al. (2018).

BEFORE YOU BEGIN

Small non-coding RNAs (sncRNAs) participate in many cellular processes, including splicing, RNA editing, mRNA degradation and/or translational inhibition (e.g., microRNAs (miRNAs)). Some of them can be secreted from the cells of origin and can be commonly found in multiple biological fluids (including plasma, urine, tears, etc.) thus potentially addressable as novel disease and therapeutic biomarkers. The absence of a clearly reported and standardized protocol to analyze the expression of sncRNAs from a low-input quantity of human peripheral blood plasma, prompted us to describe a detailed systematic and step-by-step guide to help the research community with the consistent and reproducible analysis of circulating sncRNAs. Hence, this protocol is suitable for the discovery analysis and subsequent validation of potential circulating biomarkers in many diseases.

Firstly, we describe a standardized procedure for peripheral blood draw and plasma processing and collection. These steps are of pivotal importance since the expression levels and stability of circulating sncRNAs are strongly influenced by many pre-analytical steps. Of importance, the choice of plasma EDTA over serum (Dufourd et al., 2019), centrifuge timing, force and temperature,

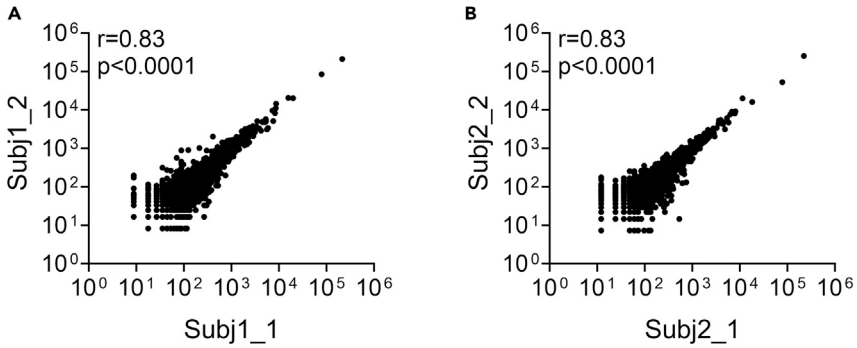

Figure 1. Reproducibility of SOP used to isolate human plasma

Reproducibility of SOP used to isolate human plasma in order to subsequently analyze sncRNAs expression is shown as correlation analysis of normalized Counts Per Million (CPM) obtained from the same healthy subject double processed (same blood draw, two different plasma aliquots) and loaded onto MiSeq platform. Spearman correlation test; $r = 0.83$; $p < 0.0001$.

elimination of contaminant cells and platelets, samples aliquoting and −80°C storage are critical for the reduction of sncRNA expression variability. Indeed, reproducibility of miRNAs expression following this Standard Operating Procedure (SOP) has been tested in a previous sncRNA-seq experiment performed on RNA extracted from multiple human plasma samples (isolated through this SOP) deriving from n=28 healthy subjects (Figure 1).

We also report the detailed steps and tips adopted to identify, quantify, and validate circulating sncRNAs from a total of 200 μL of human blood plasma using high-throughput sequencing approach followed by Stem-loop RT-Real Time qPCR validation stage.

The reported sncRNA-seq Libraries generation protocol and sequencing procedures can be performed using different Illumina Sequencer instruments and flow-cells, according to the number of samples to be analyzed and the sequencing depth needed per sample. Particularly, here we report a detailed analysis protocol using Illumina NovaSeq 6000 with XP loading workflow, which can be used to multiplex up to 384 plasma samples (using S4 flow cells) obtaining a high sequencing depth. Additionally, we report Quality Control (QC) profile of sncRNA-enriched libraries, with respect to failed libraries, as well as to set the best conditions in terms of libraries pool concentration and volumes in order to perform sncRNA-sequencing obtaining a high percentage of passing filter reads, an optimal cluster density (no over- nor hypo-clustering), as well as a high percentage of sncRNA species enrichment.

The data obtained from the sncRNAs sequencing should be confirmed through single assay validation; therefore, we reported a standardized workflow to validate sncRNA-sequencing results through multiplex Stem-Loop RT Real-Time PCR, particularly focusing on miRNAs.

In order to use this protocol at best, several steps should be anticipated and/or planned.

1. Before you start, the plasma samples should be collected using the described standard operating procedure (SOP), and stored at −80°C. The number of samples to be sequenced will determine the multiplexing magnitude and, consequently, the sequencing workflow and flow cell to be used for a specific experiment. Please note that the specific protocol described below (using Illumina SP flow cells in NovaSeq 6000 instrument) can be adopted to multiplex 64 samples/lane (total 128 samples/flow cell SP 100 cycles) thus expecting an output of $6–7 \times 10^6$ total reads/sample.
2. Prior to proceed to RNA extraction step, all the plasma samples should be analyzed for hemolysis, briefly measuring plasma absorbance at 414 nm (which should be lower than 0.3 Absorbance Unit) (Blondal et al., 2013; Shah et al., 2016).

3. Before RNA extraction, Wash solution (18 mL) of Norgen RNA extraction kit should be diluted with the right volume (42 mL) of 100 % EtOH. Before proceeding with sequencing, Sample Sheet should be prepared using Illumina Experiment Manager (IEM) software. Since no template is available to integrate the QIAGEN system into Illumina software we provide here the '.txt' template files to save into IEM program folder in order to proceed with Sample Sheet preparation.
4. Another important step is the preparation of fresh and sterile reagents the day before sequencing. In details, NaOH 1 N, Tris-HCl 400 mM pH 8.0, Tris-HCl 10mM pH 8.5 should be freshly prepared in nuclease-free water (in order to avoid nucleic acids degradation) and autoclaved the day before sequencing.

Stock solutions	Amount and storage
NaOH 1 N	Dissolve 4 g NaOH in 100 mL nuclease-free ddH_2O. Prepare it fresh each time. Store at room temperature (15°C–25°C) until the day of the sequencing (no more than 2 days).
Tris Base 1 M	Dissolve 24.2 g of Trizma Base in 200 mL nuclease-free ddH_2O. Prepare it fresh each time. Store at room temperature until the day of the sequencing (no more than 2 days).
400 mM Tris-HCl, pH 8.0	Transfer 40 mL of Tris base 1 M in a 100 mL bottle and add 50 mL of nuclease-free ddH_2O; then, add HCl until pH 8.0, measuring the volume of added HCl; finally, add nuclease-free ddH_2O up to a volume of 100 mL. Prepare it fresh each time. Store at room temperature until the day of the sequencing (no more than 2 days).
10 mM Tris-HCl, pH 8.5	Transfer 2.5 mL of 400 mM Tris HCl pH 8.0 in a 100 mL bottle and add 90 mL of nuclease-free ddH_2O; then, add NaOH until pH 8.5, measuring the volume of added NaOH; finally, add nuclease-free ddH_2O up to a volume of 100 mL. Prepare it fresh each time. Store at room temperature until the day of the sequencing (no more than 2 days).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human plasma samples	UOC Diabetologia, Azienda Ospedaliera Universitaria Senese	N/A
Chemicals, peptides, and recombinant proteins		
NaOH	Sigma-Aldrich	S8045
Trizma base	Calbiochem	648311
HCl	Sigma-Aldrich	H1758
Nuclease-Free Water (10×50 mL)	QIAGEN	Cat#129114
β-Mercaptoethanol	Sigma-Aldrich	Cat#M7522
Tris EDTA buffer, for molecular biology, DNAse, RNAse, Protease free ready to use, pH 8.0	Acros Organics	Cat#AC327345000
Critical commercial assays		
Qubit dsDNA HS Assay Kit	Thermo Fisher	Cat#Q32854
High Sensitivity DNA Kit	Agilent	Cat#5067-4626
QIAseq miRNA Library Kit (96)	QIAGEN	Cat#331505
QIAseq miRNA 96 Index IL (96)	QIAGEN	Cat#331565
PhiX Control v3	Illumina	Cat#FC-110-3001
NovaSeq 6000 SP Reagent Kit (100 cycles)	Illumina	Cat#20027464
NovaSeq XP 2-Lane Kit	Illumina	Cat#20021664
TaqMan PreAmp Master Mix	Thermo Fisher	1 mL tube (Cat#4391128)
TaqMan MicroRNA Reverse Transcription Kit	Thermo Fisher	200 reactions (Cat#4366596); 1000 reactions (Cat#4366597)

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SensiFAST Probe Lo-ROX Kit	Bioline	Cat#BIO-84020
Deposited data		
Data S1	Mendeley Data	https://doi.org/10.17632/7zcb24xbmy.1
Data S2	Mendeley Data	https://doi.org/10.17632/74msx7j24b.1
PCRNormalizator	GitHub	https://github.com/romina/PCRNormalizator
Dataset_plasma_CTR.xlsx		https://data.mendeley.com/datasets/vfj4yfffdd/draft?a=8f8b9b8a-6f69-43d2-8017-f59099fb0e02; http://dx.doi.org/10.17632/vfj4yfffdd.1
Oligonucleotides		
Individual Stem-Loop RT-TaqMan MicroRNA Assays	Thermo Fisher	4427975 (ID code is specific for each miRNA)
Software and algorithms		
2100 Expert Software (vB.02.11)	Agilent	N/A; RRID:SCR_014466
Illumina Experiment Manager Software (v1.19.1)	Illumina	N/A; RRID:SCR_021202
BaseSpace v. 1.1.0.64	Illumina	N/A; RRID:SCR_011881
GeneGlobe Data Analysis Center	QIAGEN	N/A
R/Bioconductor package NormqPCR	R/Bioconductor	N/A; RRID:SCR_001905
ExpressionSuite Software (v1.3)	Thermo Fisher	N/A; RRID:SCR_021095
Other		
Vacutainer Safety-Lok butterflies	BD Biosciences	Cat#367282
Vacutainer K2 EDTA	BD Biosciences	Cat#368861
Plasma/Serum RNA Purification Mini Kit	Norgen	Cat#55000
NucleoSpin® miRNA Plasma	MACHEREY-NAGEL	Cat#740981.10
miRNeasy Serum/Plasma Advanced Kit	QIAGEN	Cat#217204
DNA LoBind Tubes 0.5 mL	Eppendorf	Cat#0030 108.035
DNA LoBind Tubes 1.5 mL	Eppendorf	Cat#0030 108.071
DNA LoBind Tubes 2.0 mL	Eppendorf	Cat#0030 108.058
MicroAmp 96 Optical Adhesive Film	Thermo Fisher	100 covers; Cat#4311971
96 Fast PCR Plate half skirt	SARSTEDT	Cat#72.1981.202
uTIP Filtered, Low Retention XL 10µL	Biotix	Cat#M-0011-9FC
uTIP™ Filtered, Low Retention 100µL	Biotix	Cat#M-0100-9FC
uTIP™ Filtered, Low Retention 300µL	Biotix	Cat#M-0300-9FC
uTIP™ Filtered, Low Retention 1000µL	Biotix	Cat#M-1250-9FC96
Strip 8 flat caps (Xtra-Clear)	STARLAB	Cat#I1400-0900
StarTub Reagent Reservoir (PVC)	STARLAB	Cat#E2310-1000
96 PCR Plate without skirt	SARSTEDT	Cat#72.1978.202
Qubit Assay Tube Set	Thermo Fisher	Cat#Q32856
Centrifuge MiniSpin	Eppendorf	Cat#5452000010
Ministar centrifuge	VWR	Cat#521-2319
Research plus MULTI 3125 8 channels micropipette var. 30–300 µL	Eppendorf	Cat#H 3125 000 052
ViiA 7 Real-Time PCR System	Applied Biosystems	Cat#4453545
NovaSeq 6000 System	Illumina	Cat#20012850
Nanovue Uv.Vis Spectrophotometer	GE Healthcare	Cat#28956962
Research plus MULTI 3125 8 channels micropipette var. 0.5–10 µL	Eppendorf	Cat#H 3125 000 010
SimpliAmp Thermal Cycler	Applied Biosystems	Cat#A24811
DynaMag-96 Side Magnet	Invitrogen	Cat#12331D
DynaMag-2 Magnet	Invitrogen	Cat#12321D
QUBIT 3.0 spectrofluorometer	Invitrogen	Cat#Q33216
2100 Bioanalyzer Instrument	Agilent	Cat#G2939BA
IKA MS3 S36 Agilent vortex	IKA	Cat#3617036
NovaSeq Xp Flow Cell Dock	Illumina	Cat#20021663

STEP-BY-STEP METHOD DETAILS

Collection of human peripheral blood and plasma isolation and storage

Timing: 2 h maximum

This step describes how to properly isolate plasma from human whole blood in order to avoid pre-analytical biases leading to discrepancies in the downstream analyses.

1. Draw blood using BD vacutainer Safety-Lok butterflies in order to avoid hemolysis of blood samples. For sncRNA analysis, blood should be collected in K_3 or K_2-EDTA Vacutainer tubes (suggested: BD Vacutainer K_2-EDTA tubes, 3.0 mL).
2. Invert K_2-EDTA Vacutainer tubes 10 times. Blood tubes can be stored upright at room temperature (15°C–25°C) for maximum 2 h.

3. Process blood samples to isolate plasma within 2 h from blood draw, according to the following steps:
   a. Centrifuge blood containing K_2-EDTA Vacutainer tubes at 1800×*g* for 10 min at room temperature.

   Note: at this stage, centrifuging at room temperature is important in order to avoid platelet activation (Tablin et al., 2000). Activated platelets can release microvesicles containing sncRNAs thus biasing the original content of the sample (Diehl et al., 2012; Xia et al., 2018).

   Note: blood processing at 4°C appears to be attractive, because enzymatic degradation processes are reduced at low temperatures. However, platelets become activated at low temperatures (as reported in Tablin et al., 2000) and release intracellular proteins, enzymes and microvesicles-containing small RNAs, which significantly affect samples composition. Thus, blood processing at low temperatures should be performed only after platelets removal. Since one centrifugation step may not be enough for depletion of platelets below 10 cells/mL, a second centrifugation step [usually between 1,200–2,500×*g* for 15 min at room temperature (in our protocol 1200g×*g* for 20 min)] or filtration step may be required to obtain platelet poor plasma.
   b. Transfer the plasma layer into a nuclease- and pyrogen-free tube (Eppendorf style) for second centrifugation.

   Note: Plasma should be transferred in a way to avoid possible contamination. Please wear lab coats and gloves, carefully clean the work bench before and after, with an appropriate cleaner (EtOH 70% and RNAse ZAP-like solution). Please, use nuclease-free sterilized filter tips (RNase-, DNase-, DNA- and pyrogen-free) ensuring the use of clean pipettes, change the pipette filter tip after each sample when collecting plasma; be aware not to touch the buffy coat layer with pipette tip in order to avoid blood cell contamination.
   c. Collect plasma in a 2.0 mL sterile and nuclease-free tube (Eppendorf style tube) (Expected 600 μL to 1 mL of plasma for a total of 3–5 aliquots depending on the initial blood sample volume).
   d. Check plasma and note state, as shown in Figure 2.
   e. Centrifuge collected plasma samples at 1200×*g* for 20 min at 10°C.

   Note: this second centrifugation step is necessary to remove eventual contaminant cells, cell debris and remaining platelets (Shin et al., 2017; Zheng et al., 2013).
   f. Collect plasma aliquots (220 μL) into new nuclease-free sterile collection tubes and temporarily store them on ice while transferring to −80°C freezer.
   g. Store plasma aliquots at −80°C until use.

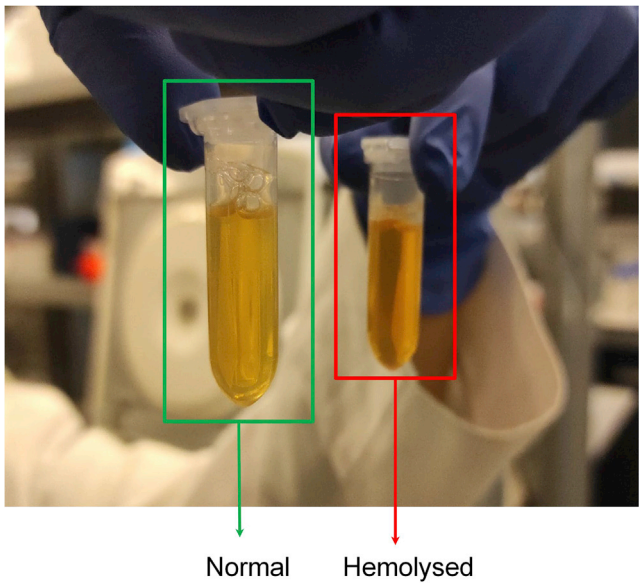

Figure 2. Plasma state

Picture showing normal and hemolyzed plasma samples collected in a 2.0 mL tube after the first centrifuge.

h. Please, refer to Figure 3 for a rapid overview of blood collection and plasma isolation protocol.

4. Following plasma isolation, hemolysis rate should be evaluated, according to the following steps (Shah et al., 2016):
   a. Using a drop spectrophotometer (e.g., Nanodrop, Nanovue, etc.), read the plasma (2 μL) absorbance at 414 nm wavelength (hemoglobin absorbance peak).
   b. If absorbance is lower than 0.072 absorbance unit (AU), the sample is not considered hemolyzed.
   c. If absorbance is comprised between 0.072 and 0.3 AU it could be low-moderate hemolyzed. In this case, consider including it in your analysis with reserve to further check the small RNAs profile upon sequencing for relevant global changes of small RNAs.
   d. If absorbance is higher than 0.3 AU, the sample is highly hemolyzed and should be excluded from further analysis.

⚠ CRITICAL: aliquots should be prepared by considering a volume of 220 μL (10% volume excess). Multiple aliquots from the same plasma sample should be prepared in order to avoid freeze-thaw cycles, which affect sncRNA stability and the following recovery and analyses. Plasma aliquots must be transferred and stored at −80°C to preserve RNA integrity (Sourvinou et al., 2013).

⚠ CRITICAL: it is important to process plasma samples within 2 hours from collection since the expression of some miRNAs could be modified in a time-dependent fashion (e.g., miR-15b and miR-191) (Khan et al., 2017).

⚠ CRITICAL: EDTA tubes are absolutely preferred to other anticoagulants. Indeed, heparin should be avoided due to its ability to inhibit enzymes used in downstream reactions, thus leading to miRNAs expression modifications. Additionally, sodium citrate is not recommended due to its ability to enhance sensitivity to hemolysis (Cui et al., 2011; Khan et al., 2017).

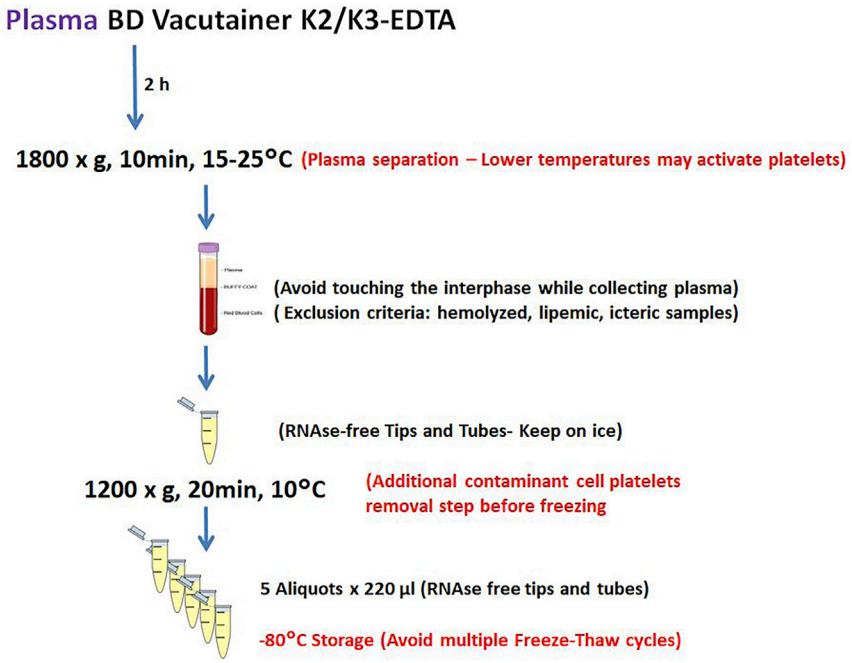

Figure 3. Blood collection and processing workflow

RNA extraction

Timing: 1 h 30 min

RNA extraction is performed from 200 µL of plasma using Plasma/Serum RNA Purification Mini Kit from Norgen (see Key Resource Table) following manual's instructions (https://norgenbiotek.com/sites/default/files/resources/Plasma-Serum-RNA-Purification-Kit-PI55000-5.pdf) with minor modifications.

Optimal and best performing RNA extraction method was selected among three different candidate protocols following an explorative sncRNA sequencing experiment performed on plasma samples deriving from a healthy subject. Briefly, total RNA was extracted from 200 µL of human plasma using n=3 different RNA extraction kits (200 µL/each kit): a) Advanced miRNeasy, b) Machery-Nagel and c) Norgen. sncRNA libraries were generated through QIASEQ miRNA library kit, and subsequently sequenced using MiSeqDX platform. In details, data analyses showed that all RNA extraction kits were able to generate sncRNA libraries (Figure 4A). Sequencing primary analysis revealed that Machery-Nagel resulted in 4.07×10^6reads/sample, Norgen libraries returned 3.91×10^6 reads/sample and Advanced MiRNeasy resulted in 3.55×10^6reads/sample (Figure 4B). Among total reads, Machery-Nagel resulted in 26% of mapped miRNAs (normalized ratio on total reads), Norgen returned 44% miRNAs composition rate and Advanced MiRNeasy returned 49% miRNAs (Figure 4C). In this set of experiments, both Norgen and Advanced MiRNeasy kits outperformed similarly, then they can be both adopted in this workflow. In our standardized protocol, we adopted Norgen kit and further described its specific procedure below.

5. Thaw plasma samples (200 µL) on ice.

   Note: Complete thawing of a 200 µL plasma aliquot on ice requires approximately 30 minutes.

6. Prepare complete Lysis Buffer A: warm Lysis Buffer A (included in the Norgen kit) at 60°C for at least 20 min to dissolve eventual precipitates. When at room temperature, add 10 µL of β-Mercaptoethanol for each mL of Lysis Buffer A.

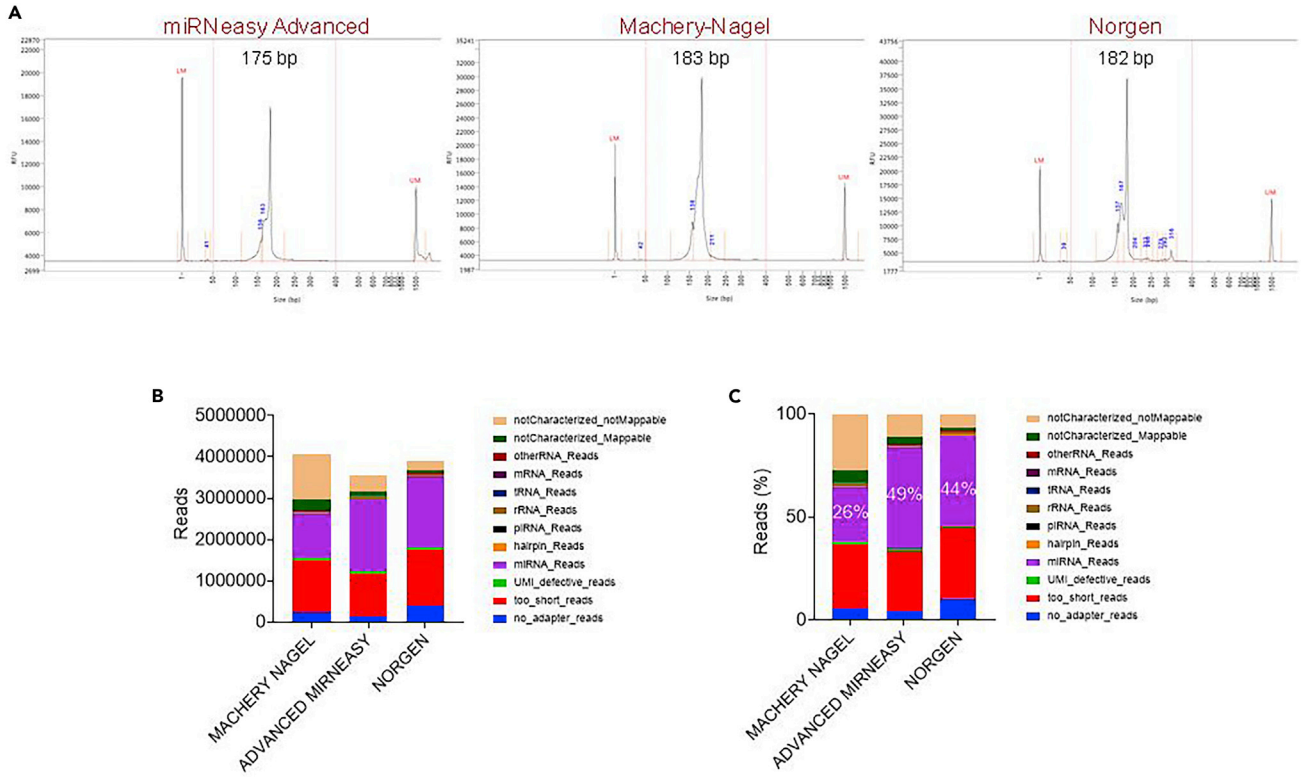

Figure 4. RNA extraction kits

Figure showing the results of small RNA sequencing on RNA extracted from plasma (200 μL) of a single human healthy subject with n=3 different RNA extraction kits. (A) Bioanalyzer electropherograms showing profiles of libraries generated using miRNeasy Advanced protocol (Average peak 175 bp), Machery-Nagel protocol (Average peak 183 bp) and Norgen protocol (Average peak 182 bp). Sequencing primary analysis revealed that (B) Machery-Nagel RNA libraries resulted in 4.07×10^6 reads; Advanced miRNeasy RNA libraries resulted in 3.55×10^6 reads and Norgen RNA libraries resulted in 3.91×10^6 reads. Among total reads (C) Machery Nagel enriched for 26% of miRNAs, Advanced miRNeasy enriched for 49% miRNAs, and Norgen enriched for 44% miRNAs. This latter was chosen as RNA extraction protocol.

⚠ CRITICAL: Please ensure that Lysis Buffer A is completely resuspended, and no further precipitate is visible before proceeding. An uncomplete resuspension of Lysis Buffer A as well as non-addition of β-Mercaptoethanol could result into an inefficient lysis leading to a low RNA recovery. See Figure 5 for a complete resuspended Lysis Buffer A working solution.

7. Centrifuge plasma samples 400×*g* for 2 min at room temperature before processing.

   Note: this step is critical to further remove cell debris and larger particles from plasma samples.

8. Transfer 200 μL of centrifuged plasma sample into a new 2.0 mL tube (sterile, nuclease-free, pyrogen-free).
9. Add a volume of 600 μL of Lysis Buffer A + β-Mercaptoethanol. Mix well by vortexing for 10 s.
10. Add 800 μL of 96–100% ethanol. Mix well by vortexing for 10 s.
11. Transfer 650 μL of the resulting mixture into a Micro Spin column (included in Norgen kit). Centrifuge 3,300×*g* for 2 min. Discard the flow-through and reassemble the spin column with its collection tube.
12. Repeat this step twice more, until the whole mixture has been transferred to the Micro Spin column.

Figure 5. Norgen Lysis Buffer A

Norgen Lysis Buffer A working solution should appear clear, transparent and without precipitates before proceeding to RNA extraction.

13. Add 400 μL of Wash Solution A to the column and centrifuge for 30 s at 3,300×*g*; discard the flow-through and reassemble the spin column with its collection tube.
14. Repeat this step twice more, for a total of three washes.
15. Spin the column, empty, for 2 min at 13,000×*g*. Discard the collection tube.
16. Transfer the spin column to a fresh 1.5 mL elution tube.
17. Elute by adding 20 μL of Elution Solution A to the column and let stand at room temperature for 2 min.
18. Centrifuge for 1 min at 400×*g*, followed by 2 min at 5,800×*g*.
19. Transfer the eluted volume back to the column and let stand at room temperature for 2 min.
20. Centrifuge for 1 min at 400×*g*, followed by 2 min at 5,800×*g*.
21. Approximately, a final eluted volume of 18 μL should be present in the tube.
22. Maintain the tube on ice until storage at −80°C.

Note: it is not helpful to quantify extracted total RNA from 200 μl of human plasma samples since the absolute quantity is generally too low for commonly used RNA quantification methods (e.g., Spectrophotometer, Spectrofluorometer or capillary electrophoresis) (Figure 6).

SncRNA library preparation and quality controls (QCs)

Timing: 2 days for 8–16 samples/each lab operator

In this section, we describe a systematic protocol to prepare sncRNA libraries using Qiaseq miRNAs Library kit (see Key Resource Table) following manufacturer's instructions (https://www.qiagen.com/us/resources/resourcedetail?id=f0b9d117-f8c6-4825-9069-3236ed65521d) with modifications. This library preparation kit was chosen based on the large use of QIASeq MicroRNA kit reported in literature, where it is reported as the most performant kit (Heinicke et al., 2020) (Wong et al., 2019), as well as based on a previous set of experiments on murine plasma samples, aimed at comparing different sncRNAs library preparation kits, and showing that QIASeq returned the best results in terms of quality and yield (data not shown).

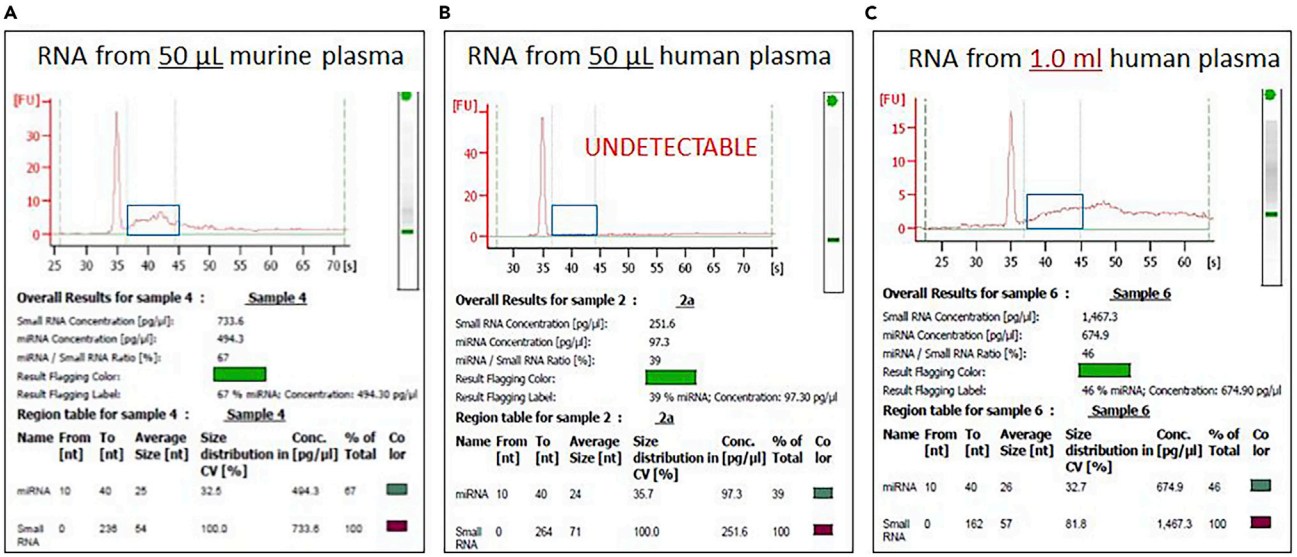

Figure 6. Bioanalyzer 2100 electropherograms

Bioanalyzer 2100 electropherograms showing (A) the enrichment of sncRNA species (blue rectangle) in RNA extracted from 50 µL of murine plasma, (B) undetectability (below detection limit and similar to a negative CTR) of sncRNA species in RNA extracted from 50 µL of human plasma (blue rectangle). In order to consistently quantify (ng) circulating sncRNA species in human plasma samples, RNA should be extracted at least from 1.0 mL of human plasma (C).

The following protocol (see Summarizing scheme below) is designed for optimal preparation of eight samples/reaction. Based on our experience we suggest processing no more than sixteen samples/reaction, since some steps require quick and straightforward handling; however, here we consider libraries preparations standardized on 8 samples/reaction. Delays in completing some steps (e.g., cDNA cleanup) significantly affect the quality of cDNA libraries preparation.

sncRNA libraries preparation summarizing scheme

Order	Step/reaction	Timing
	3′ Adapter Ligation	2 h
	5′ Adapter Ligation	1 h 15 min
	Beads Preparation/Activation	45 min
	Reverse Transcription Initiation	30 min
	Reverse Transcription Reaction	1 h 45 min
	cDNA CleanUp	1 h
–	*Pause Point*	–
(7)	PCR cDNA Amplification	1 h 45 min
(8)	PCR CleanUp	1 h 30 min
(9)	Libraries Concentration (QUBIT)	20 min
(10)	Libraires Fragments size (2100 Bioanalyzer)	1 h

23. Perform 3′ Adapter ligation reaction (for 8 samples) as follows:
    a. Thaw at room temperature: 3′ Adapter, 3′ Buffer, 2× Ligation Activator (located at −20°C) and then resuspend the reagents by flicking and briefly centrifuging the tubes.
    b. Thaw RNA samples on ice, resuspend them by flicking, and briefly centrifuge the tubes; then put RNA samples on ice.
    c. Dilute 3′ Adapter 1:5 in a 0.5 mL tube: 2 µL 3′ Adapter + 8 µL nuclease-free water (keep at room temperature). Spin down, resuspend up and down (15 times) with pipette volume set to 8 µL and spin down again.

d. Prepare 3′ Adapter ligation mix on ice and add components according to the following order:

Component	Volume (1 sample)	Volume (for 8 samples[a])
QIAseq miRNA NGS 3′ Adapter (1:5)	1 μL	8.4 μL
QIAseq miRNA NGS RI	1 μL	8.4 μL
QIAseq miRNA NGS 3′ Ligase	1 μL	8.4 μL
QIAseq miRNA NGS 3′ Buffer	2 μL	16.8 μL
2× miRNA Ligation Activator	10 μL	84 μL
Template RNA (added in step 23 h.) [b]	5 μL[§]	/
TOTAL VOLUME	20 μL	126 μL

[a]Final volumes include a 5% excess calculation.
[b]**Note:** The input RNA volume is 5 μL/18 μL of total eluted RNA per sample. Increasing input RNA quantity by performing a Speedvac (10 minutes room temperature) step (18 μL→5 μL) of the total eluted RNA, in order to load the entire volume of extracted RNA, did not improve sncRNA libraries yield, QC, sequencing metrics and reads distribution of Small RNA species (Figure 7).

e. Briefly centrifuge, pipette up and down 15 times with a micropipette set to 120 μL, centrifuge again.
f. Prepare 2 rows of a 96-well plate (Figure 8A). Use the right-sided row of the well plate and mark it with a pen marker. Place them into a cooler (Figure 8B).
g. Add 15 μL/well of MIX to the bottom of the well.
h. Add 5 μL of RNA sample per well.
i. Resuspend 15 times with multichannel micropipette set to a 10 μL volume.
j. Cap with a row of strips and briefly spin down.
k. Start the reaction according to the following thermal cycler steps:

Cycling conditions	
Temperature	Time
28°C	1 h
65°C	20 min
4°C	5 min (at least)
4°C	hold

⚠ CRITICAL: this reaction is very viscous due to the Ligation activator reagent. Pipette slowly and carefully.

⚠ CRITICAL: the efficiency of the thermal cycler may affect the yield of the libraries. For the present protocol we adopted the SimpliAmp thermal cycler (see Key Resource Table) with the following specifications: 96-well 0.2 mL Veriflex Block; Max block Ramp rate: 4°C/second; Max sample ramp rate: 3°C/sec; Temperature accuracy: ± 0.25°C (35.0°C–99.9°C); Temperature Uniformity: <0.5°C.

⚠ CRITICAL: in order to avoid contamination between samples and libraries, it is essential to clean gloves with 70% EtOH every time you cap and uncork the wells with the strips. Maintain this caution for all libraries preparation steps.

Note: for all reactions, enzymes should be thawed at the time of use and immediately put on ice.

24. Perform 5′ Adapter ligation reaction (for 8 samples) as follows:

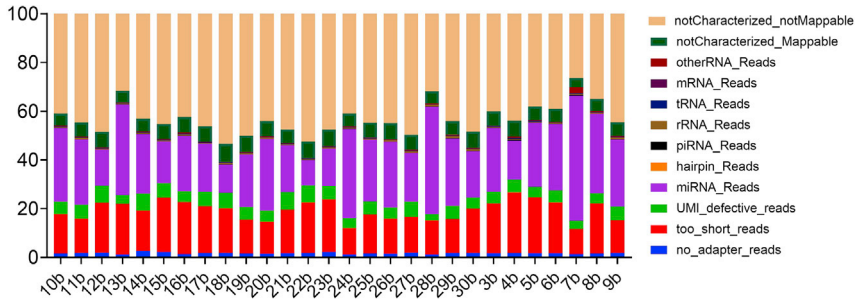

Figure 7. Reads distribution

Stacked bars showing reads distribution of small RNA libraries generated from 5 µL of RNA concentrated through Speedvac vacuum centrifuge extracted from plasma of n=28 healthy subjects. MiRNAs are the more represented species of small RNA, corresponding to a mean of 23.6%.

a. Thaw at room temperature the 5′ Adapter and 5′ Buffer reagents. Bring them to room temperature. Resuspend and spin down.
b. Dilute 5′ Adapter 1:2.5 in a 0.5 mL tube: 4 µL 5′ Adapter + 6 µL nuclease-free water. Resuspend 15 times by pipette with volume set to 8 µL and spin down again.
c. Prepare reaction mix on ice and add components according to the following order:
d. Pipette 15 times with multichannel pipette volume set to 140 µL.

Component	Volume (1 sample)	Volume (8 samples)[a]
Nuclease-free water	15 µL	126 µL
QIAseq miRNA NGS 5′ Adapter (1:2.5)	2 µL	16.8 µL
QIAseq miRNA NGS RI	1 µL	8.4 µL
QIAseq miRNA NGS 5′ Ligase	1 µL	8.4 µL
QIAseq miRNA NGS 5′ Buffer	1 µL	8.4 µL
TOTAL VOLUME	20 µL	168 µL

aFinal volumes include a 5% excess calculation.

e. Remove 3′ Ligation reactions from thermal cycler, briefly spin down and place back into cooler.
f. Uncork the strip from 3′ Ligation reactions and add 20 µL of MIX to each well.
g. Resuspend 15 times with 30–300 µL multichannel pipette with volume set to 35 µL.
h. Cap again with a new strip, briefly spin down and start the reaction according to the following thermal cycler steps:

Cycling conditions	
Temperature	Time
28°C	30 min
65°C	20 min
4°C	5 min (at least)
4°C	Hold

⚠ CRITICAL: in order to avoid freezing of samples, remember (from this reaction onward) to put 96-well thermal cooler at room temperature few minutes before starting the reaction.

25. Prepare and activate beads (for 8 samples) as follows:
    a. Take DynaMag2 Magnet (16 positions for 2.0 mL tubes).
    b. Take Beads bottle and Beads Binding Buffer bottle (both at +4°C).
    c. Consider 400 µL beads/sample×8 = 3,200 µL to distribute in 2 × 2.0 mL tubes (1,600 µL/each tube).

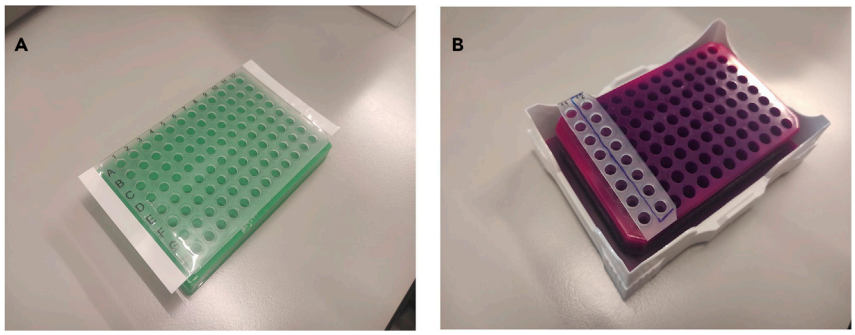

Figure 8. Plates preparation

(A) Before cutting the 2 rows from the plate, apply a PCR adhesive film on it in order to avoid contamination.

(B) 2 rows of a 96 well plate prepared for 3′ Adapter ligation reaction for 8 samples. Place the 2 rows in a thermo cooler rack in order to keep a low temperature of the reactions.

d. Vortex bottle beads for about 1 min.
e. Add 1,600 μL of beads into the first tube by making two 800 μL pipettings (pipette slowly).
f. Vortex the beads bottle again for about 30 s and add another 1,600 μL into the second tube by performing two pipettings of 800 μL (pipette slowly).
g. Place the 2 tubes into the Dynamag2 magnet. The beads will migrate in about 15–20 min.
h. Briefly vortex the Bead Binding Buffer.
i. Remove the supernatant from the beads with the p1000 set to 800 μL, very slowly and with the tubes still on the magnet. Be sure not to remove the beads and make sure the beads have fully migrated.
j. Remove the tubes from the magnet and add 600 μL of Beads Binding Buffer (150 μL Beads Binding Buffer/sample: ×4/tube = 600 μL /each tube from 2.0 mL) directly onto the beads.
k. Vortex the two tubes for about 1 min at high speed and briefly spin down.
l. Place the two tubes back on the magnet for about 10–12 min or until the beads have migrated.
m. Remove the supernatant very slowly avoiding taking beads away (check the tip).
n. Vortex the Beads Binding Buffer.
o. Pull the tubes away from the magnet.
p. Add 1,600 μL of Bead Binding Buffer/sample (400 μL Beads Binding Buffer/sample: ×4/× tube = 1,600 μL/each tube from 2.0 mL) directly onto the beads.
q. Vortex until the beads are homogeneously resuspended (example in Figure 9) and store at +4°C.

⚠ CRITICAL: in order to avoid carry away beads thus modifying their concentration, leave a minimal liquid volume in the first step of supernatant elimination following beads complete migration. Also avoid letting beads go into the cap; if they do go into the cap briefly spin down.

Note: prepared and activated beads can be stored at +4°C and used within 1 week.

26. Perform Reverse transcription (RT) initiation reaction:
    a. Thaw at room temperature RT Initiator reagent, resuspend by flicking and briefly spin down.
    b. Thaw the following reagents: RT Buffer, RT Primer, resuspend and briefly spin down.
    c. Take the 5′ Ligation reaction out of the thermal cycler and briefly spin down. Store into a thermal cooler.
    d. Remove the strips from the wells and add 2 μL of RT Initiator/Sample.
    e. Resuspend 15 times with multichannel micropipette set to 35 μL.

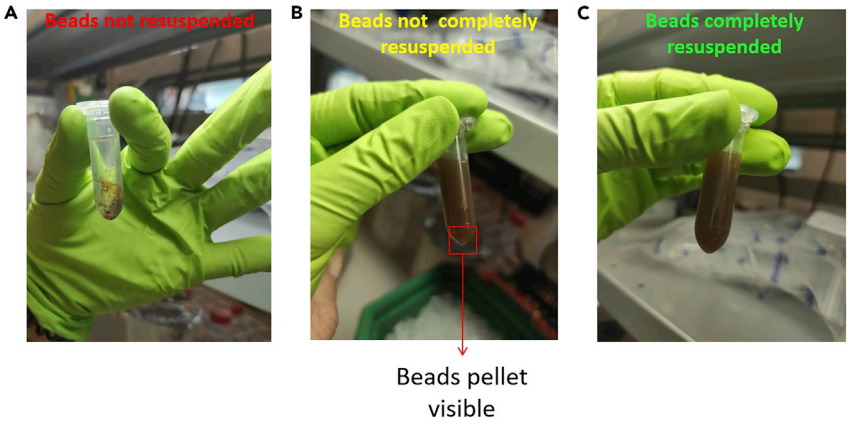

Figure 9. Beads resuspension state

Pictures show different levels of beads resuspension.

(A) Beads not resuspended not usable for cleanup.

(B) Beads not completely resuspended showing a small pellet, not usable for cleanup.

(C) Beads completely resuspended usable for cleanup.

f. Put new strips and briefly spin down; start the reaction according to the following thermal cycler steps:

Cycling conditions	
Temperature	Time
75°C	2 min
70°C	2 min
65°C	2 min
60°C	2 min
55°C	2 min
37°C	5 min
25°C	5 min
4°C	hold

Note: RT Buffer and RT Primer could be thawed together with RT initiator; indeed, since RT initiation reaction takes circa 20 minutes, in this time the operator can start to dilute RT primers and to prepare RT mix for the following reaction.

27. Perform RT reaction:
    a. Dilute RT Primer 1:5 in nuclease-free water. For 8 samples: 4 µL RT Primers + 16 µL nuclease-free water; resuspend for 15 times and briefly spin down.
    b. Prepare RT MIX (for 8 samples) and maintain it on ice:

Component	Volume (1 sample)	Volume (8 samples)[a]
QIAseq RT primer 1:5	2 µL	16.8 µL
Nuclease-free water	2 µL	16.8 µL
QIAseq miRNA RT buffer	12 µL	100.8 µL
QIAseq miRNA RI	1 µL	8.4 µL
QIAseq miRNA RT	1 µL	8.4 µL
TOTAL VOLUME	18 µL	151.2 µL

aFinal volumes include a 5% excess calculation.

c. Resuspend 15 times with a multichannel pipette with volume set to 140 μL.
d. Remove RT Initiator reactions from the thermal cycler, spin down, place it back into a cooler and remove strips.
e. Add 18 μL of RT mix/sample.
f. Resuspend 15 times with multichannel pipette set to 53 μL.
g. Strip the tubes, spin down and start the reaction according to the following thermal cycler steps:

Cycling conditions

Temperature	Time
50°C	1 h
70°C	15 min
4°C	5 min (at least)
4°C	Hold

28. Perform cDNA cleanup:
a. Freshly prepare 80% EtOH in nuclease-free sterile water.
b. Vortex beads for at least 1 min at a low vortex speed.
c. From now on work at room temperature. Add 143 μL of previously activated beads/well. Vortex beads every 2 wells.
d. Resuspend 20 times with multichannel micropipette set at 190 μL and incubate 5 min at room temperature.
e. Place samples to the 96-well magnet and do not remove until the end of the procedure.
f. Incubate 15 min to allow complete migration of the beads.
g. Add 5.0 mL of 80% EtOH into the reservoirs and remove supernatant with multichannel micropipette set at 190 μL. Aspirate slowly and leave a small volume.
h. Add 200 μL 80% EtOH/sample.
i. Recover all 80% EtOH with volume set to 200 μL and repeat step h. and i.
j. Cap with strips. Very briefly spin down and put back on magnet 1 min.
k. Aspirate remaining volume with 10 μL multichannel pipette (several times if needed).
l. Perform air dry 10–12 min (beads drying shown in Figure 10).
m. Add 17 μL of nuclease-free water directly onto the beads (without touching them with the tip).
n. Remove from magnet and resuspend by pipetting 20 times.
o. Incubate 2 min at room temperature.
p. Place back on magnet for 2 min.
q. Recover 15 μL/sample and transfer to another sterile well of 96 wells plate (as previously shown in Figure 8).

⚠ CRITICAL: do not pass with anything (pipette, hands, etc.) over the reservoir containing EtOH; if EtOH is contaminated, also libraries will be contaminated.

⚠ CRITICAL: prepare 10 mL of 80% EtOH, of which 5.0 mL are used for the first cleanup and the remaining 5.0 mL are used for the second cleanup. However, immediately following usage, EtOH-remaining-volume must be para-filmed, otherwise its concentration will be reduced due to evaporation.

⚠ CRITICAL: air dry is a critical step, since the not complete dry (which means EtOH-remaining volume) could influence the following sequencing analyses; on the other side, too much dried beads will result in a low libraries recovery and low libraries concentrations.

Pause point: the eluted material can be stored at −20°C before to proceed to the next step the next day. In our experience, stopping at this step results in a higher libraries concentration (Average 120 nM vs average 30 nM) (Example in Figure 11).

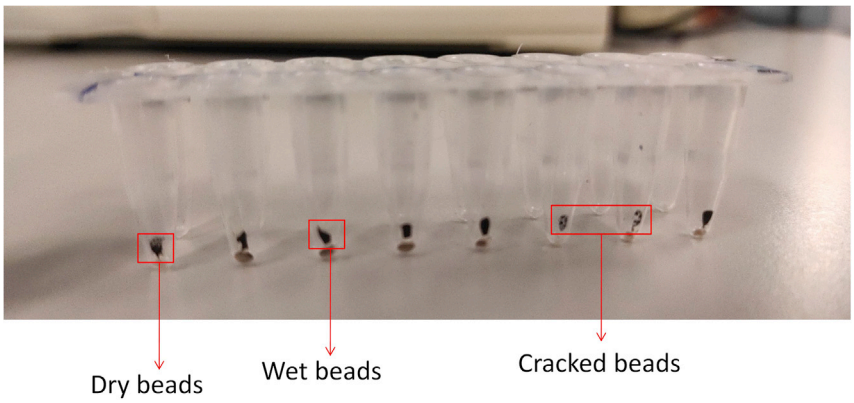

Figure 10. Beads drying state

This picture shows 3 different levels of beads drying. From right to left: those named 'cracked beads' are too much dried and cannot further be considered to elute the sample; those named 'wet beads' have not yet completely dried, thus containing ethanol which can influence sequencing analyses; those named 'dry beads' are completely dried but not cracked and are perfect to be hydrated with water to perform elution step.

29. Perform cDNA amplification:
    a. Thaw at room temperature the Amplification Library Buffer reagent and nuclease-free water (troubleshooting: problem 1).
    b. Thaw Index Plate and place it into the cooler for few minutes. Cut the wells of interest (use fine scissors - e.g., from surgery) (Figure 12).
    c. Thaw cDNA and place it into the cooler.
    d. Prepare MIX (8 samples) in the following order:

Component	Volume (1 sample)	Volume (8 samples)
QIAseq Library buffer	8 µL	67.2 µL
Nuclease-free water	15.5 µL	125.24 µL
QIAseq Hot Start Taq	1.5 µL	12.12 µL
TOTAL VOLUME	25 µL	204.56 µL

    e. Resuspend by pipette 15 times with volume set at 185 µL.
    f. Add 25 µL of MIX to each well of the cut index well (DO NOT PIPETTE).
    g. Spin down cDNA samples.
    h. Transfer 15 µL of each cDNA sample to its respective position in the index row.
    i. Resuspend by pipette 25 times with pipette set to 34 µL. Do not spin (no new strips can be inserted).
    j. With the tips still into the wells, set multichannel pipette volume to 42 µL. Transfer to a new well taking care to recover all the volume (repeat with multichannel at 10 µL if necessary).
    k. Put new strips onto the wells, spin down and start the reaction according to the following thermal cycler steps:

Cycling conditions		
Step	**Temperature**	**Time**
Hold	95°C	15 min
Start 3-step cycling: 22 cycles		
Denaturation	95°C	15 s
Annealing	60°C	30 s
Extension	72°C	15 s
Stop 3-step cycling		
Hold	72°C	2 min
Hold	4°C	5 min (at least)
Hold	4°C	Storage

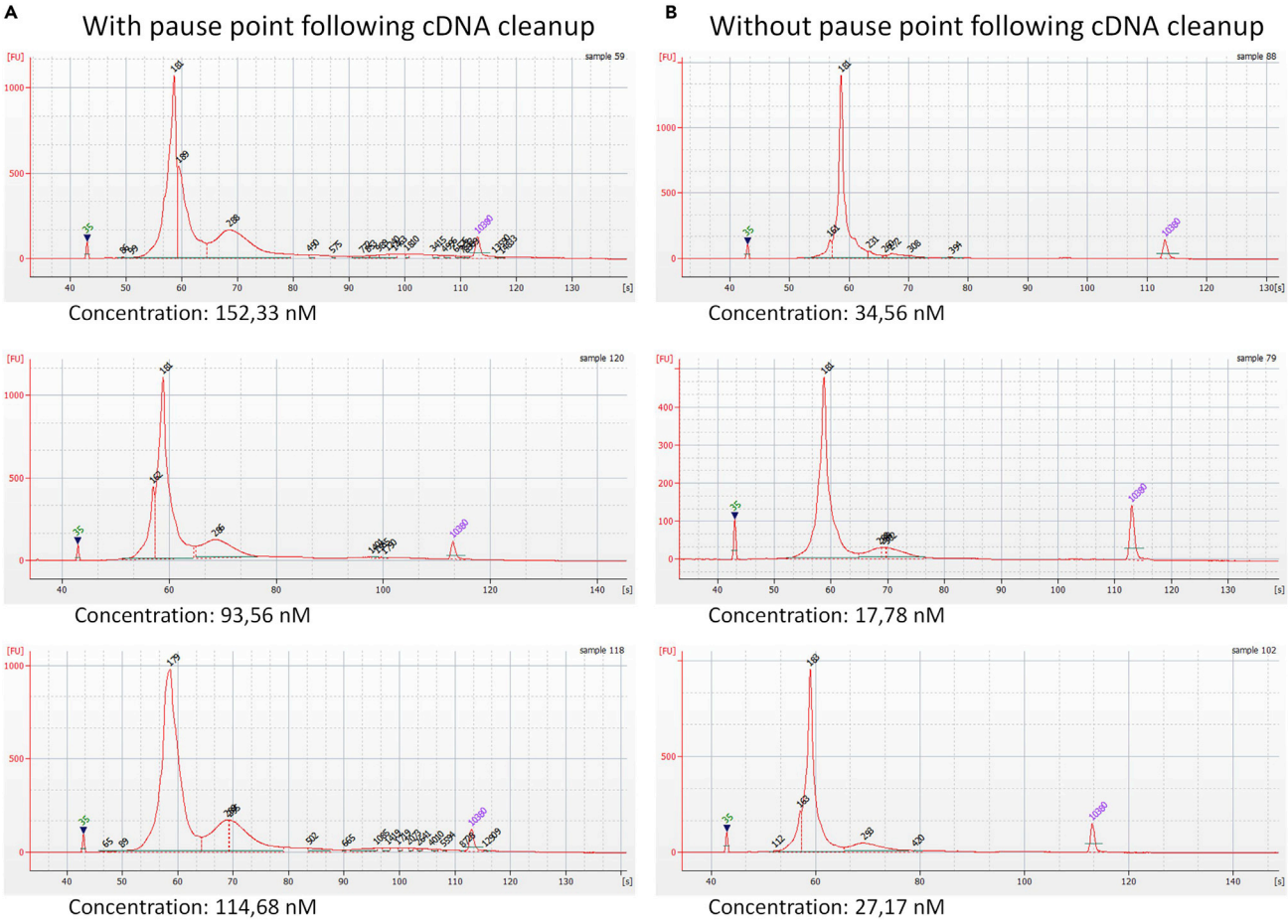

Figure 11. Quality control of libraries following pause point step

Examples of (A) n=3 electropherograms in which storage at −20°C pause point has been performed following cDNA cleanup and of (B) n=3 electropherograms in which storage at −20°C pause point has not been performed following cDNA cleanup. While libraries profiles were acceptable in both cases, the concentration is strongly reduced without pause point application (mean value w/ −20°C storage: 119,6 nM vs 79,45 Mean w/o −20°C storage).

Note: the direction of the index row is indicated by the presence of numbers at the bottom.

⚠ CRITICAL: index plate should be left in thermal cooler for some minutes, otherwise the wells could break while being cut.

⚠ CRITICAL: indexes corresponding to a specific sample must be annotated, in order to associate each index to a sample for the following sequencing.

⚠ CRITICAL: it is necessary (although not planned by manual's instructions) to transfer the reaction into new wells since caps of provided index plate are not airtight, and, consequently, the reaction could evaporate.

30. Perform amplification product cleanup:
    a. Pull out prepared and activated beads from +4°C at least 5 min before starting.
    b. Vortex the beads for 1 min to resuspend them and briefly spin down.
    c. Remove amplification reactions from thermal cycler, briefly spin down and put them in a rack at room temperature.

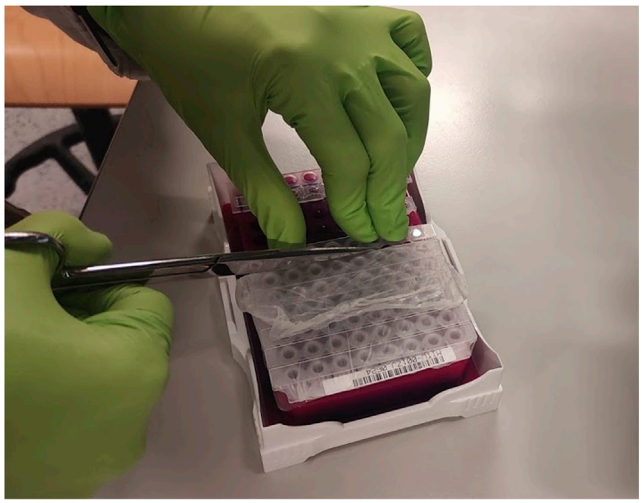

Figure 12. Index plate wells cutting
Picture of index plate wells cutting.

d. Vortex beads again (vortex high speed for few seconds and then low speed for 1 min).
e. From now on work at room temperature.
f. Add 37.5 μL beads/sample (Vortex beads every two samples).
g. Resuspend 15 times with multichannel pipette set at 70 μL and incubate 5 min at room temperature.
h. Transfer to the magnet and incubate at least 5 min or until complete migration is achieved.
i. Recover all the supernatant by setting multichannel micropipette to 80 μL and transfer to new sterile wells placed on the rack. Carefully inspect whether no libraries volume is left in the wells: if necessary, recover the remaining volume with 10 μL multichannel pipette, then throw away beads.
j. Vortex beads bottle as previously done and add 65 μL beads/sample by vortexing every 2 samples.
k. Resuspend 15 times with multichannel pipette set at 120 μL and incubate 5 min at room temperature.
l. Transfer to magnet and incubate for at least 10 min.
m. Transfer 5.0 mL 80% EtOH into a new sterile reservoir.
n. Eliminate supernatant from the beads, taking care not to take away the beads, setting pipette to 135 μL.
o. Add 200 μL 80% EtOH/sample on the opposite side of the beads.
p. Remove 80% EtOH and add 200 μL 80% EtOH again.
q. Remove all the volume, cap with new strips and briefly (no more than 1 s) spin down.
r. Place back the plate on magnet and remove any residual volume.
s. Air Dry 10–12 min on the magnet.
t. Add 17 μL nuclease-free water on the beads and remove from magnet.
u. Resuspend about 20 times, setting pipette to 15 μL and incubate for 2 min at room temperature.
v. Transfer the plate again onto the magnet for 2 min at room temperature.
w. Recover 15 μL supernatant and transfer to a new sterile plate.
x. Maintain samples into the cooler or store the samples at −20°C.

31. Measure libraries concentration using QUBIT 3.0 spectrofluorometer:
    a. Work with light off.
    b. For each sample prepare working solution by adding 1 μL DNA high sensitivity QUBIT fluorescent reagent to 199 μL DNA high sensitivity QUBIT buffer (if you are going to analyze more samples, always calculate an extra sample for working solution preparation).

c. Transfer 199 μL working solution into a QUBIT 0.5 mL new sterile tube and add 1 μL of each library; to read the standards, transfer 190 μL working solution into a QUBIT 0.5 mL new sterile tube and add 10 μL of each standard (#1 and #2).
d. Resuspend, spin down and incubate 2 min at room temperature.
e. Read samples concentration with the program DNA → High sensitivity, selecting 1 μL as volume (troubleshooting: problem 2).

Note: it is not necessary to read standards every time. It is needed when more than one week from the last read passed, or if last time you have read samples with another kit (RNA, protein, etc.).

32. Perform libraries quality control (QC) through Bioanalyzer 2100 by using dsDNA high sensitivity kit:
    a. Bring to room temperature all reagents (ladder, marker, gel matrix and dye) at least 30 min before chip loading.
    b. Prepare gel dye mix by adding 15 μL blue dye into 300 μL gel matrix, vortex 30 s and briefly spin down.
    c. Load the prepared gel dye mix onto dedicated filter and centrifuge 2,240×*g* 10 min.
    d. Prepare empty chip for electrodes wash by adding 450 μL of nuclease-free water.
    e. Remove and discard the filter from gel dye mix prepared tube.
    f. Load 9 μL gel dye mix into the G chip well and perform chip priming (Figure 13).
    g. Load 9 μL gel dye mix into the G chip wells (n=3) (Figure 13).
    h. Load 5 μL marker in every well apart for G wells (n=4).
    i. Load 1 μL samples into the appropriate wells and 1 μL ladder into the well illustrated in Figure 13.
    j. Wash electrodes by applying wash chip (loaded with nuclease-free water) for 1 min, closing the instrument door; then allow electrodes to dry by leaving open the instrument door for 1 min.
    k. Meanwhile vortex the loaded chip for 1 min into the appropriate IKA MS3 vortex At 2400 RPM.
    l. Load the chip onto the instrument.
    m. Start the run.
    n. For specific results see "expected outcomes" section (troubleshooting: problem 3)

⚠ CRITICAL: chip priming is an essential and critical step; plunger should return to at least 0.3 mL (usually 0.5 mL of the syringe plug scale), otherwise repeat chip priming on a new chip. Another critical step is sample loading; avoid bubbles and, above all, quickly load samples following marker loading, otherwise reagents will wear out in the meantime, resulting in a wrong run.

Pause point: at this point libraries can be directly normalized or stored at −20°C (stable for several weeks).

Note: prepared gel-dye mix is sufficient for five chips loading and can be used within 6 weeks from date of preparation.

33. Calculate concentration, normalize and pooling libraries
    a. Use the following formula to calculate nM concentration of each Library sample:

$$\frac{QUBIT\ concentration\left[\frac{ng}{\mu l}\right]}{(Average\ peak\ of\ small\ RNAs\ of\ Bioanalyzer\ 2100)\ x\ 660} \times 10^6$$

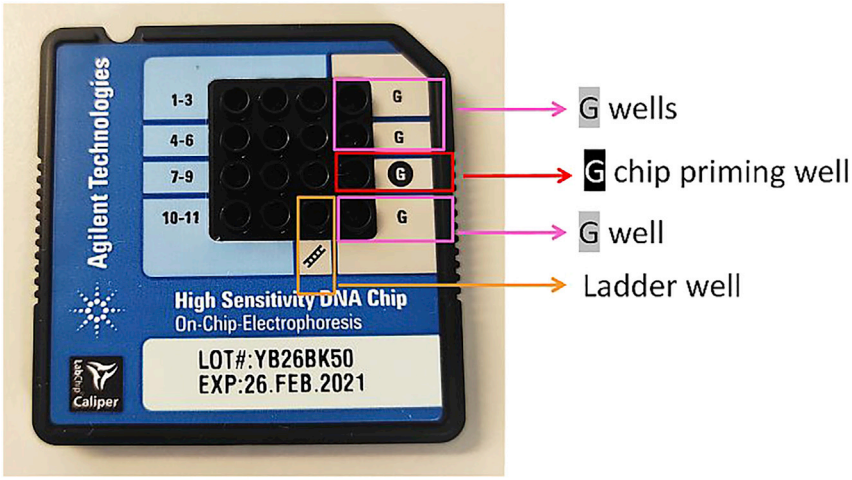

Figure 13. Bioanalyzer 2100 dsDNA chip

Picture showing Bioanalyzer 2100 dsDNA chip and different wells to perform chip priming and samples loading. In details, G wells (as indicated in the main text) are marked in pink, G well (as indicated in the main text) for chip priming is marked in red, and ladder well is marked in orange.

Note: to select the correct Average peak of small RNAs please refer to the electropherogram and instructions shown in Figure 14.

b. Normalize libraries concentration by dilution in nuclease-free water until 2.0 nM and performing an intermediate step to 10 nM if libraries are concentrated more than 20 nM.
c. Read Libraries concentration using QUBIT 3.0 after each intermediate or final dilution step and then proceed with further dilution or directly to pooling step.
d. Pool all normalized libraries using at least 3 μL of each diluted/normalized library.
e. Read again the concentration obtained from the libraries multiplexed pool using QUBIT 3.0 spectrofluorometer as previously described.
f. If needed adjust the final concentration of the libraries pool (2 nM) by further diluting it with nuclease-free water.

⚠ CRITICAL: for libraries pooling, at least 3 μL of each library are required since smaller volumes result in a higher final concentration error. For libraries normalization consider an error range of ±0.3 nM (from 1.7 to 2.3 nM). A high variability between sample concentration could affect the equilibrium among different samples and the quality of small RNA sequencing (troubleshooting: problem 4).

Sequencing sample sheet preparation

Timing: variable, depending on samples number

34. Create an Illumina Account on Illumina website (URL: https://login.illumina.com).
35. Download the latest version of Illumina Experiment Manager (IEM) software (v1.19.1) at the following link: https://emea.support.illumina.com/sequencing/sequencing_software/experiment_manager/downloads.htmL.
36. Install Illumina Experiment Manager software following instructions.
37. Following installation, a folder named 'Illumina' will be generated in PC storage disk 'C:Program Files (x86)'.
38. The 'Illumina' folder contains another folder named 'Illumina Experiment Manager' in which there are other sub-folders: 'Applications', 'Genomes', 'IndexKits' and 'SamplePrepKits'.

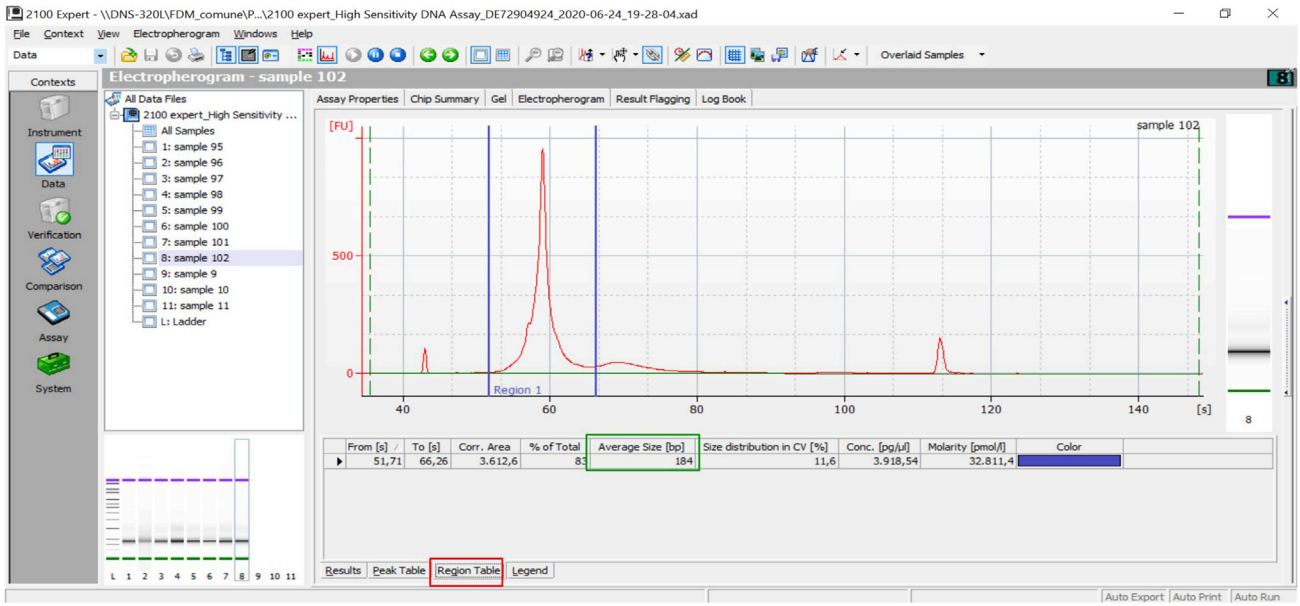

Figure 14. Selection of region of interest in 2100 Expert software

In 2100 Expert software, the region of interest can be selected in the sheet 'Region Table' (red rectangle; selection of the region should consider the main peak starting from the beginning of this peak to the beginning of the *hump* at 290–300 bp (vertical blue lines). Following this selection, the Average Size in bp will be returned (green rectangle).

39. In 'SamplePrepKits' folder, paste the '.txt' file attached to this paper as Data S1 (Deposited as a dataset on Mendeley Data with the https://doi.org/10.17632/7zcb24xbmy.1, URL: https://data.mendeley.com/datasets/7zcb24xbmy/1).
40. In 'Applications' folder, paste the '.txt' file attached to this paper as Data S2 (Deposited as a dataset on Mendeley Data with the https://doi.org/10.17632/74msx7j24b.1, URL: https://data.mendeley.com/datasets/74msx7j24b/1).
41. Open IEM software and select 'Create Sample Plate'.
42. Select QIASEQ small RNA index adapters and press NEXT; rename the plate and press NEXT.
43. Rename your samples ID and assign to each sample the specific QIASEQ index used during amplification reaction; press finish.
44. Save the plate template in a folder easy to find (the suggested folder is hidden, and it is difficult to find).

Note: if you are going to sequence more than 96 samples you have to prepare more than one plate.

⚠ CRITICAL: be careful not to use the same QIAGEN index for two or more samples that will be sequenced in the same pool/lane of the Flow Cell! IEM will return an error, and Sample Sheet will not be generated.

45. Re-open IEM software and select 'Create Sample Sheet'
46. Then select 'NovaSeq' instrument and press NEXT
47. Choose 'NovaSeq FASTQ only' and press NEXT
48. Select the right Run parameters as indicated in Figure 15 and press NEXT:

Note: the option 'Adapter Trimming' indicates the trimming of the 3'Adapter, while 'Adapter Trimming Read 2' indicates the trimming of the 5' Adapter.

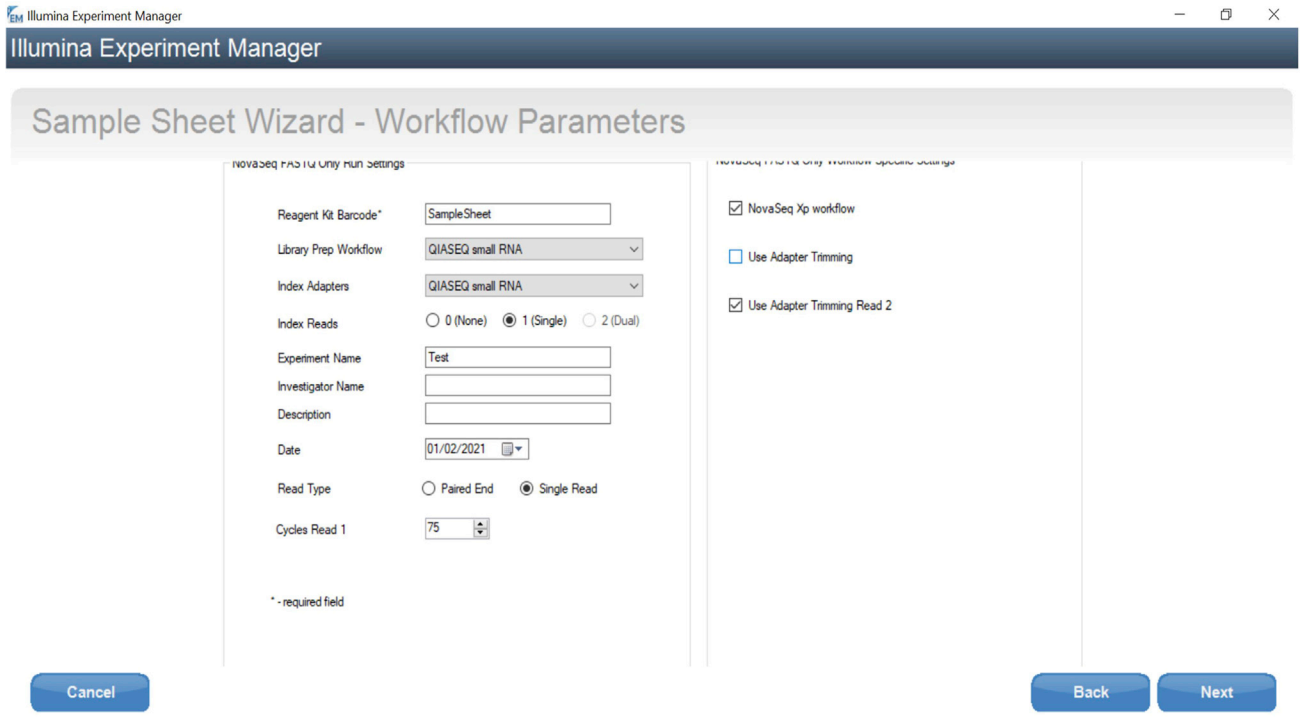

Figure 15. Sample Sheet parameters
Sample Sheet parameters to be set on Illumina Experiment Manager software.

⚠ CRITICAL: if you plan to perform reads mapping and primary analysis through GeneGlobe QIAGEN online software, deselect 'Adapter Trimming', since QIAGEN software algorithm requires 3′ Adapter to recognize a correct processed '.fastq' file. Indeed, if 3′Adapters sequences are previously trimmed, Gene Globe analysis generates an error, and the files cannot be further processed.

49. Press 'Select Plate': in the box at right all the samples you listed in the previously prepared plate will appear.
50. Select all the samples that will be sequenced in the same lane of the Flow Cell and select the lane; then press 'Add selected samples' and 'Finish'.
51. Save the prepared Sample Sheet in '.csv' format.
52. DO NOT OPEN the Sample Sheet in Excel when the software asks.

Note: if it is necessary to open and check the Sample Sheet, open it as '.txt' file, otherwise it will not be recognized later on by NovaSeq 6000 instrument.

NovaSeq 6000 sncRNA sequencing using NovaSeq XP protocol

Timing: 1 day

The day before sequencing prepare and autoclave:

NaOH 1N in nuclease-free water.
Tris-HCl 400 mM pH 8.0 in nuclease-free water.
Tris-HCl 10 mM pH 8.5 in nuclease-free water.

Table 1. Table listing libraries pool concentrations, volumes, and reagents specifications adopted for small RNA seq on NovaSeq 6000 XP protocol depending on the used flow cell

Flow cell	Lanes number	Pooled library concentration	Final loading concentration	PhyX %	Pooled library volume	0.2 N NaOH	400 mM Tris HCl, pH 8	ExAmp Master mix volume	Final loading volume
SP/S1	2	0.95 nM	175 pM	6%	18 µL (15.75 µL library pool + 2.25 µL of PhyX 0.25 nM)	4 µL	5 µL	210 µL (126 µL DPX1, 18 µL DPX2, 66 µL DPX3)	90 µL/lane (27 µL denatured library + 63 µL ExAmp M. Mix)
S2	2	0.95 nM	175 pM	6%	22 µL (19.25 µL library pool + 2.75 µL of PhyX 0.25 nM)	5 µL	6 µL	210 µL (126 µL DPX1, 18 µLDPX2, 66 µL DPX3)	110 µL/lane (33 µL denatured library + 77 µL ExAmp M. Mix)
S4	4	0.95 nM	175 pM	6%	30 µL (26.25 µL library pool + 3.75 µL of PhyX 0.25 nM)	7 µL	8 µL	525 µL (315 µL DPX1, 45 µL DPX2, 165 µL DPX3)	150 µL/lane (45 µL denatured library + 105 µL ExAmp M. Mix)

Note: following protocol and data have been set up using SP flow cell. In order to optimize the protocol on the other flow cells (S1, S2 or S4), please refer to Table 1.

53. Thaw SBS and Cluster cartridges by placing them in a room temperature water bath (19°C–25°C) by submerging approximately halfway for 3 h.

Note: Use cartridges within 4 hours from the complete thawing.

54. Dilute libraries and PhyX control and pool them (the following steps refer to SP/S1 Flow cell loading concentration and volumes):
    a. Thaw Library pool and PhiX 10 nM on ice.
    b. Prepare 3 clean sterile 1.5 mL nuclease-free tubes.
    c. Final loading concentration of Library pools is 175 pM.
    d. Normalize/dilute Library pool as following (Final concentration: 0.95 nM): 9.5 µL of 2 nM Library pool+10.5 µL TrisHCl 10 mM pH 8.5.
    e. Dilute 10 nM PhiX control until 0.25 nM (1:40) in TrisHCl 10 mM pH 8.5: 1 µL of 10 nM PhiX + 39 µL TrisHCl pH 8.5.
    f. Mix 2.25 µL of diluted PhiX control with 15.75 µL of 0.95 nM normalized Library pool (PhiX control 6 %, Final Volume=18 µL).
    g. Before to proceed to denaturation:
        i. Thaw DPX1, DPX2 and DPX3 ExAmp reagents at room temperature for 15 min, then place them on ice.
        ii. Put Flow Cell at room temperature for 30 min before loading.

Note: use Flow Cell within 12 hours!

55. Denature libraries and PhyX pool:
    a. Dilute 1N NaOH solution (to be diluted immediately before the use) until 0.2 N in nuclease-free water by mixing 2 µL of 1N NaOH with 8 µL of nuclease-free water.
    b. Denature normalized/diluted Library pool (A and B) and PhiX control mix (Vol=18 µL) by adding 4 µL of 0.2 N NaOH to each mix; then cap the tube, briefly vortex and centrifuge 280×*g* for 1 min; allow the denaturation by incubating the sample 8 min at room temperature.
    c. Add 5 µL of 400 mM TrisHCl pH 8.0; then cap the tube, briefly vortex and centrifuge 280×*g* for 1 min to obtain a final volume of 27 µL.
    d. Place the denatured Library pool on ice.

56. Prepare Flow cell:

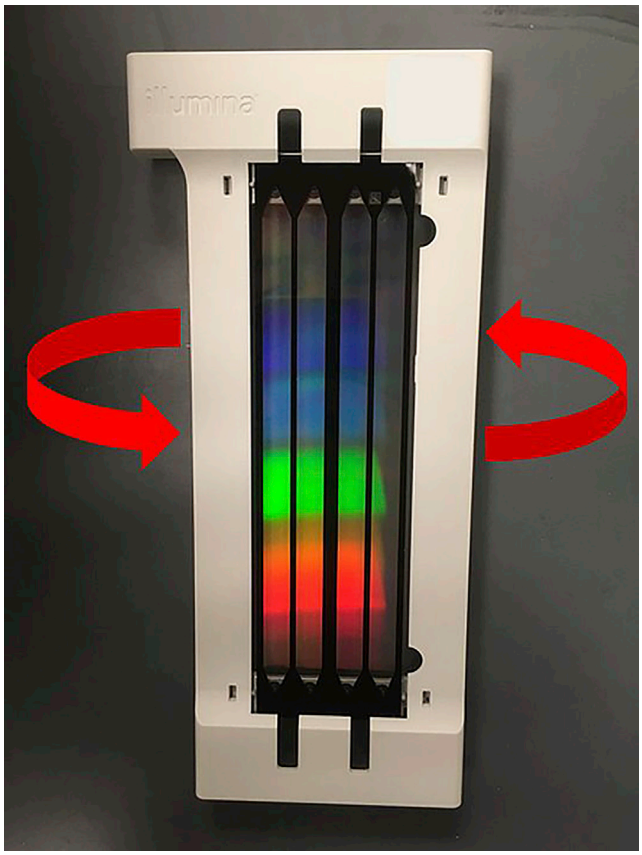

Figure 16. Flow cell inversion movement
Movement by which flow cell should be inverted.

a. Place the NovaSeq Xp flow cell dock (see Key Resource Table) on a flat surface and keep it until the loading onto the instrument.
b. Wear a new clean pair of gloves not to contaminate the glass of flow cell dock and remove the clear plastic by peel opening the foil from the corner tab.
c. Remove the flow cell dock from the package by touching it by the sides not to touch the glass.
d. Inspect the dock is free from particulate; if particulate is present clean the surface with a lint-free alcohol wipe.
e. Invert the flow cell so that the top surface faces downward (with the movement indicated in Figure 16).

Note: in this case, pool A contains n=28 libraries whose preparation has been performed using 5/18 μL of RNA; pool B contains n=28 libraries whose preparation has been performed using 5 μL of concentrated RNA from the total 18 μL of eluted RNA (test performed as reported in 3′ Adapter Ligation note).

f. Slip the outlet end of the flow cell under the bracket and place it onto the dock.
g. Load the Novaseq Xp manifold over the inlet end of the flow cell (be careful that the manifold fit securely).
h. Close the clamp to block flow cell and manifold and seal the gaskets.

57. Prepare ExAmp Master Mix:
    a. Briefly vortex DPX1, DPX2 and DPX3 reagents and briefly centrifuge it.

b. In a sterile nuclease-free 1.7 mL tube mix: 126 µL of DPX1, 18 µL of DPX2 and 66 µL of DPX3 to obtain a final volume of 210 µL of ExAmp Master Mix (the mix could appear cloudy: it is normal).
c. Mix the reagent by slowly pipetting with a p200 micropipette and vortex 30 s.
d. Centrifuge the mix at 280×*g* for 1 min and immediately proceed to the loading.

⚠ CRITICAL: DPX2 and even more DPX3 are very viscous reagents: be careful to take the right volume.

58. Load libraries onto the Flow Cell:
    a. Add 63 µL ExAmp Master Mix in the tube containing 27 µL of denatured Library pool, vortex for 30 s and centrifuge at 280×*g* for 1 min.
    b. Slowly load 80 µL of mixed Sample + ExAmp mix to each Novaseq Xp manifold well; avoid touching the filter.
    c. Wait 2 min to allow the mixture to reach the opposite side of the lane (do not touch the flow cell!).

Note: the manifold wells are reversed, so be sure to load the right sample in the right well (as indicated in Figure 17).

Note: Do not tilt the flow cell.

59. Prepare thawed SBS and Cluster cartridges
    a. Carefully inspect the cartridges that should be free of ice and water.
    b. Invert the cartridges 10 times to mix reagents, and gently tap the cartridges to break all bubbles.
60. Load empty Library tube
    a. Uncap the Library tube provided with the NovaSeq 6000 reagent kit.
    b. Insert in position #8 the uncapped empty Library tube.
61. Set up a sequencing run
    a. From the Home screen select Sequence and then select A or B (A+B to select a dual flow cell run).
    b. Select OK to acknowledge the warning and open the flow cell door.
62. Load the flow cell onto the instrument
    a. Inspect the flow cell stage that should be clean; if particulate is present clean the entire stage, including glass, fluidic interface and flow cell holder with alcohol and dry with lint-free wipe.
    b. Remove the flow cell from the dock:
        i. Open the clamp that blocks flow cell and manifold.
        ii. Carefully remove and discard the manifold.
        iii. Clean the flow cell with alcohol and dry with lint-free wipe if liquid drops are present.
        iv. Remove the flow cell by grasping it from the sides.
        v. Clean the gaskets (without touching them) with alcohol and dry with lint-free wipe if liquid drops are present.
        vi. Invert the flow cell as previously described in Figure 16.
        vii. Clean the dock with alcohol and dry with lint-free wipe if particulate is present.
    c. Align the flow cell over the four raised clamps and place it on the flow cell stage.
    d. Select Close Flow Cell door (the flow cell ID will appear on the screen).
63. Load the SBS and Cluster Cartridges:
    a. Open the liquid compartment doors and then open the reagent chiller door.
    b. Load the cartridges (the SBS cartridge with the gray label into the left position, and the cluster cartridges with the orange label into the right position).

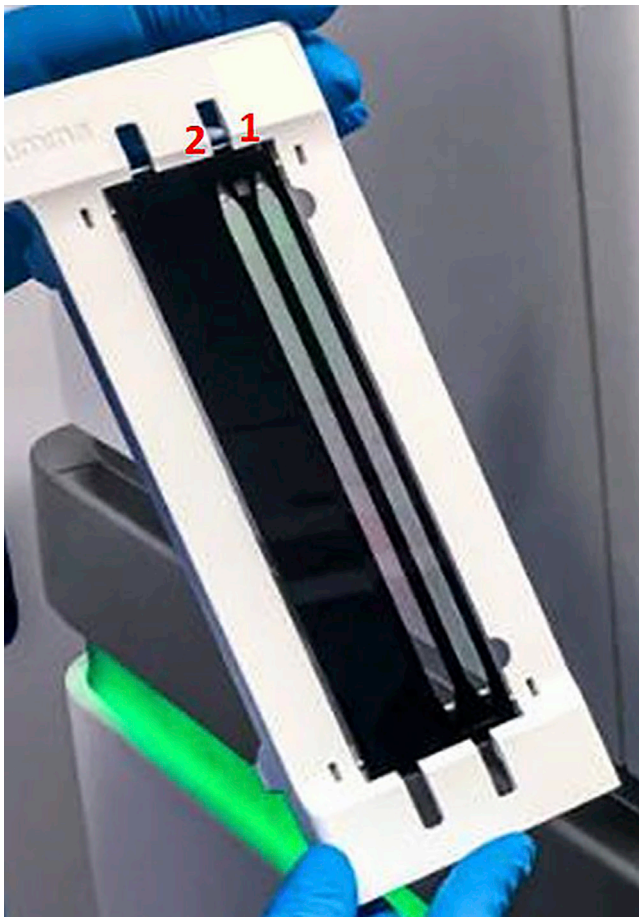

Figure 17. Flow cell inverted order
Figure representing the inverted order of the two lanes of the flow cell.

c. Side the drawer into the chiller and close the reagent chiller door (The Library tube ID will appear on the screen).

64. Load the buffer cartridges and start run:
    a. Pull the metal handle to open the buffer drawer.
    b. Place the buffer cartridge into the buffer drawer so that the Illumina label faces the front of the drawer and align the cartridge.
    c. Select the button Log In to sign in to BaseSpace Sequence Hub.
    d. To send run data to BaseSpace Sequence Hub for remote monitoring and data analysis, select Run Monitoring and Storage (this option requires a Sample Sheet).
    e. Enter your BaseSpace Sequence Hub username and password and select Sign In.
    f. If prompted, select a workgroup to upload run data to and select Run Setup.
    g. Select a workflow type (in this case NovaSeq Xp) and rename the run.
    h. Enter the number of cycles and index length.
    Read1 76 cycles
    Index1 8
    Read2 0
    Index2 0
    i. Select Advanced options, select Sample Sheet and Browse to upload your ready Sample Sheet (.csv).

j. Select Start Run and wait about 5 min until the pre-run check is completed.

Note: although the sequencing run is 1×75, when setting the parameters in the instrument, one more cycle should be considered; indeed, one more cycle is required for instrument phasing and prephasing calculations (Kircher et al., 2011).

⚠ CRITICAL: In order to correctly perform data analysis using BaseSpace and then Gene-Globe QIAGEN miRNA quantification:

k. Use Illumina Experiment Manager (IEM) to generate the Sample Sheet.
l. Rename the Sample Sheet as "SampleSheet.csv" in order to be recognized by BaseSpace.
m. Do not open the file using excel or other software. If needed, use only notepad.
n. Be sure to include the sequences of 5′ or 3′ Adapter you want to be Trimmed out by Base-Space.
o. Since Gene Globe QIAGEN miRNA quantification online tool have a Trimming tool included and instructed to trim out 3′ Adapters, if your FastQ files contain reads already trimmed out for both adapters, the algorithm generates an error (">95% Reads of Sample X did not have custom sequencing adapter") and does not proceed to the analysis.

Single assay validation of selected miRNAs

Timing: variable, depending on number of miRNAs to validate

Since sncRNA sequencing analysis required 5 out of 18 µL of total eluted RNA extracted from plasma samples, the same RNA aliquot can be used to perform the subsequent validation. The following validation protocol is based on the use of Stem-Loop RT Primers and Taqman Assays RT-Real Time PCR (Chen et al., 2005).

65. Perform Multiplex Custom miRNAs Reverse Transcriptase (RT) reaction:
    a. Prepare custom Stem-Loop RT primer pool by diluting each 5× RT primer 1:100 (ex: 5 µL of each primer in a total volume of 500 µL of TE 1×; 5 µL of primer 1 + 5 µL of primer 2 + 5 µL of primer 3 + 485 µL of TE 1×) in TE 1×. Up to 96 RT Stem-Loop primers can be multiplexed.
    b. Prepare the RT reaction mix on ice in a 1.5 mL tube as follows:

Component	X 1 sample
RT primer pool	6 µL
dNTPs 100 mM	0.3 µL
Multiscribe Reverse Transcriptase	3 µL
10× RT buffer	1.5 µL
RNase inhibitor	0.19 µL
Nuclease-free water	1.01 µL
TOTAL	12 µL

    c. Mix thoroughly and briefly spin down the tube.
    d. In a 0.2 or 0.1 mL tube (depending on the thermal cycler), add 12 µL of RT reaction mix into each tube and add 3 µL of total RNA per reaction into each tube containing RT reaction mix for a total reaction volume of 15 µL.
    e. Resuspend and briefly spin down the samples. Perform RT at the following thermal cycling conditions:

Cycling conditions		
Step	Temperature	Time
Hold	16°C	30 min
Hold	42°C	30 min
Hold	85°C	5 min
Hold	4°C	Storage

Important: include a negative control reaction (3 μL nuclease-free water reaction) in order to evaluate potential cross-primers effects and external/carry-over contaminations (troubleshooting: problem 5).

66. Perform Custom miRNAs PreAmplification:
    a. Prepare custom PreAmp primer pool by diluting each 20× TM primer 1:100 (ex: 5 μL of each primer in a total volume of 500 μL of TE 1×) in TE1×. Up to 96 TM primers can be multiplexed.
    b. Prepare the reaction mix in a 1.5 mL tube as follows:

Component	Volume (1 sample)
RT product	2.5 μL
Taqman PreAmp Master Mix 2×	12.5 μL
PreAmp Primer Pool	3.75 μL
Nuclease-free water	6.25 μL
TOTAL	25 μL

    c. In a 0.2 or 0.1 mL tube (depending on the thermal cycler), transfer 22.5 μL of the PreAmp reaction mix and 2.5 μL of each RT product into each well or tube.
    d. Start run according to the following thermal cycler conditions:

Cycling conditions		
Step	Temperature	Time
Hold	95°C	10 min
Hold	55°C	2 min
Hold	72°C	2 min
Start 2-step cycling (12 cycles)		
Denaturation	95°C	15 s
Annealing	60°C	4 min
Stop 2-step cycling		
Hold	99.9°C	10 min
Hold	4°C	Storage

    e. Remove the samples from the thermal cycler and put on ice.
    f. Dilute samples 1:8 by adding 175 μL of 0.1× TE pH 8.0 to each tube. This is the diluted PreAmp product (Final Volume = 200 μL) that can be used for qRT Real-Time PCR.

Important: include a negative control reaction (2.5 μL nuclease-free water reaction) in order to evaluate potential cross-primers effects and external/carry-over contaminations independent from RT reactions.

67. Perform Real Time PCR:
    a. Prepare a mix for each microRNA as follows:

Component	Volume (1 sample)
Sensifast probe Lo-ROX Master Mix	10 μL
Nuclease-free water	7 μL
Taqman microRNA assay	1 μL
TOTAL	18 μL

b. Load 2 μL of PreAmplified cDNA (diluted 1:8) in each well of a 96 well plate in duplicate.
c. Add 18 μL of the previously prepared mix.
d. Start run according to the following thermal cycling conditions:

Cycling conditions		
Step	Temperature	Time
Hold	95°C	2 min
Start 2-step cycling (40 cycles)		
Denaturation	95°C	10 s
Annealing	60°C	20 s
Stop 2-step cycling		
Hold	4°C	Storage

Note: if a higher output is needed for low expressed microRNAs, a higher amount of cDNA can be loaded considering that: to obtain 1 Ct decrease you need to load 2× cDNA; to obtain 3.32 Ct decrease you need to load 10× cDNA.

Note: we used a Viia7 qPCR instrument (Thermo Fisher), by performing a fast run protocol.

Note: always consider to add a No Template Control (NTC) sample, loading 2 μL of nuclease-free water (instead of sample) and 18 μL of mix.

EXPECTED OUTCOMES

sncRNAs (including miRNAs, snRNAs, snoRNAs, tsRNAs and piRNAs) can be secreted by their cells of origin and can be found in multiple biological fluids. Therefore, they can be used as biomarkers for disease staging, disease diagnosis, patient stratification, therapeutic prognosis and monitoring. Indeed, the possibility that their circulating expression levels may reflect in-situ tissue/cells alteration(s) in a given disease attracted much interest in biomarkers discovery field.

The need for novel biomarkers is particularly evident in highly heterogeneous and complex diseases (such as immune-related disorders, autoimmune diseases and cancer), characterized also by a long and variable prodromic stage. This is the case for several heterogeneous disorders such as cancer and autoimmune diseases, including type 1 diabetes.

Multiple studies have evaluated plasma or serum circulating miRNAs as potential biomarkers for patient stratification and type 1 diabetes progression (Dotta et al., 2018). However, lack of concordance on identified candidate sncRNAs/miRNAs represent an issue, impeding a compelling validation of clinically useful biomarkers. Most of the observed discordance can be imputed to differences in samples processing, dissimilarities among high-throughput analysis and validation platforms, data analysis and normalization strategies.

Here, based on our experience and on tested methods and platforms, we report a systematic protocol for the consistent and reproducible analysis of plasma circulating sncRNAs, starting from peripheral blood draw to discovery and sncRNA validation (with focus on miRNAs).

We initially describe a standardized procedure to collect and process blood samples in order to obtain plasma. Such procedure represents a pivotal step for the successful and consistent evaluation of circulating sncRNAs.

Then, we adopted a sncRNA sequencing approach, which allows the detection of the entire spectrum of sncRNAs (15–60 nts) in plasma samples. Tips and quality controls are also clearly reported in order to

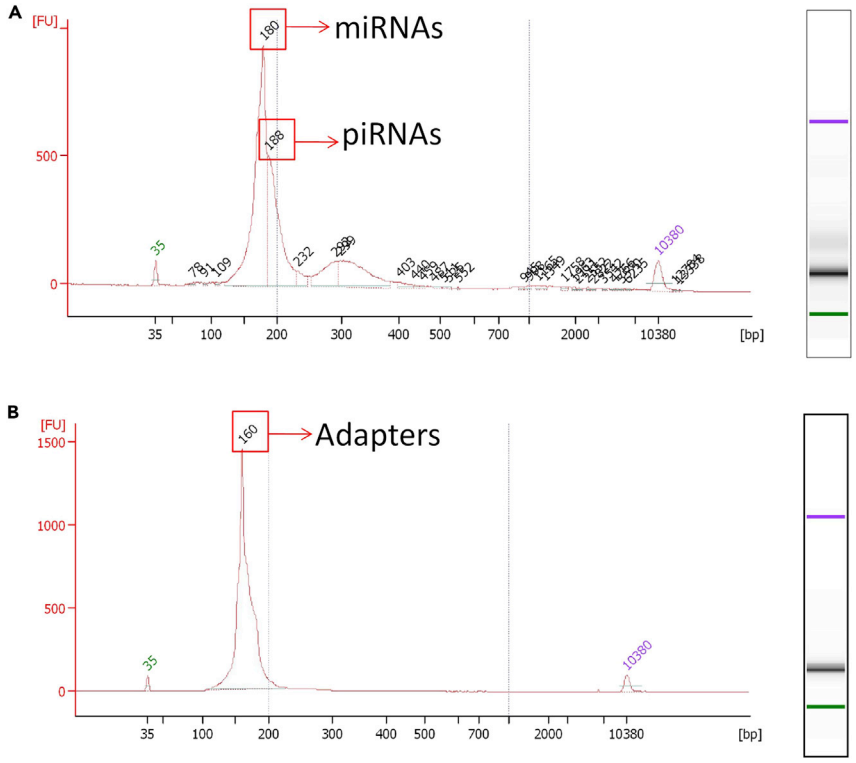

Figure 18. Examples of successful and failed libraries electropherograms

The electropherogram in (A) shows both the peaks corresponding to miRNAs (180 bp) and to piRNAs (188 bp). No adapter peaks are visible, denoting high quality of the generated library. The electropherogram in (B) shows a failed library only enriched in adapters (160 bp) (Sample 5a).

correctly evaluate a successful cDNA library preparation before sequencing step. Using capillary electrophoresis, a 15–25 nts sncRNA-enriched library should show a peak around 180 bp, with the possibility of an additional peak at 188 bp, potentially indicating piRNAs as shown in an example electropherogram in Figure 18A. We reported examples of failed samples (3 out of 28), mainly showing adapter peaks (example in Figure 18B), corresponding to a peak of 150-160 bp, indicating the length of adapters lacking 15–25 nts sncRNA sequence. As a matter of fact, following sequencing analyses, reads distribution of these 3 libraries showed a lower miRNAs amount (average 10%) and higher too short reads rate (average 74%) with respect to those libraries enriched in miRNAs and other species of sncRNAs (average miRNA amount 25%–30%; average too short reads amount 20%) (Figure 19).

The sequencing step was performed using a NovaSeq 6000 Illumina Instrument adopting the lowest capacity flow cell (SP). Of note, we underline the possibility to multiplex up to 384 samples in a single run using a XP workflow-loading mode and multiple indexes. Based on our results, sequencing metrics showed a Q30% score of 96.5% and 85.2% clusters (reads) passing filter, resulting into a total of 5.44×10^8 reads. Cluster passing filter reads resulted evenly distributed among samples, resulting in a mean of 1.5×10^7 reads in each sample (Figure 20A), which represent a high depth that allows also discovery analyses. Among total reads in each sample, around 3.0×10^6 were represented by miRNAs (Figure 20B), 1.34×10^4 by piRNAs (Figure 20C) and 1.78×10^4 by tRNA fragments (Figure 20D). Collectively these results demonstrate that multiple small RNA species can be consistently analyzed using the above protocol.

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: variable (usually 1 week)

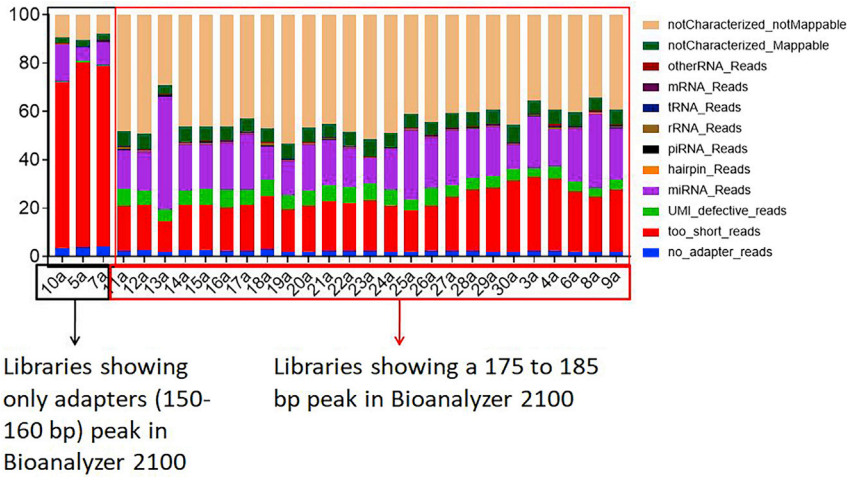

Figure 19. Stacked bars graph showing small RNA species reads distribution among samples which libraries were prepared from 5/18 μL eluted from RNA extraction

On the left, samples 10a, 5a and 7a show a high percentage of too short reads corresponding to adapters as observed in Bionalyzer libraries profile. On the right, the other 25 samples show a particular enrichment in miRNAs reads (Average 20%).

Base calling and demultiplexing of NovaSeq 6000 output was performed on BaseSpace platform from Illumina, using GenerateFASTQ plugin version 1.1.0.64 (https://basespace.illumina.com).

The primary quantification analysis aimed at mapping and identifying the different sncRNA species was performed by submitting raw FASTQ files to QIASeq miRNA analysis according to automated pipeline of QIAGEN GeneGlobe Data Analysis Center portal (http://ngsdataanalysis2.qiagen.com/QIAseqmiRNA).

In more details:

1. Reads are first processed by trimming of 3′ Adapter and low-quality bases using cutadapt (cutadapt.readthedocs.io/en/stable/guide.html).
2. Then, reads with less than 16 bp insert sequences (*'too_short_reads'*), reads with UMI sequences shorter than 10 bp (*'UMI_defective_reads'*) and reads with no adapter sequence (*'no_adapter_reads'*) are discarded.
3. Remaining sequences are analyzed following a stepwise alignment strategy to identify the different sncRNA species by mapping to specific databases.
   a. Sequences are aligned sequentially to miRbase mature, miRBase hairpin, noncoding RNA, mRNA and other RNA using bowtie (bowtiebio.sourceforge.net/index.shtml) with no mismatches allowed.
   b. Then, a second mapping to miRBase mature is carried on unmapped sequences tolerating up to two mismatches. miRBase V.21 is used for miRNA, where entries with identical or near-identical sequences are combined. piRNABank database is used for piRNA identification.
   c. All remaining unmapped sequences are aligned to the human genome (Genome Reference Consortium GRCh38) to identify possible novel miRNA molecules.
   d. For each sample, all reads assigned to each species-specific RNA category are counted, and the associated UMIs are aggregated to count unique molecules.
   e. Read and UMI counts are presented in an output Excel® file reporting "miR_piRNA", "tRNA", "otherRNA" sheets, respectively. Remaining reads are reported in "not Characterized_mappable" sheet, if they align to the genome, or "notCharacterized_notMappable" if they don't.

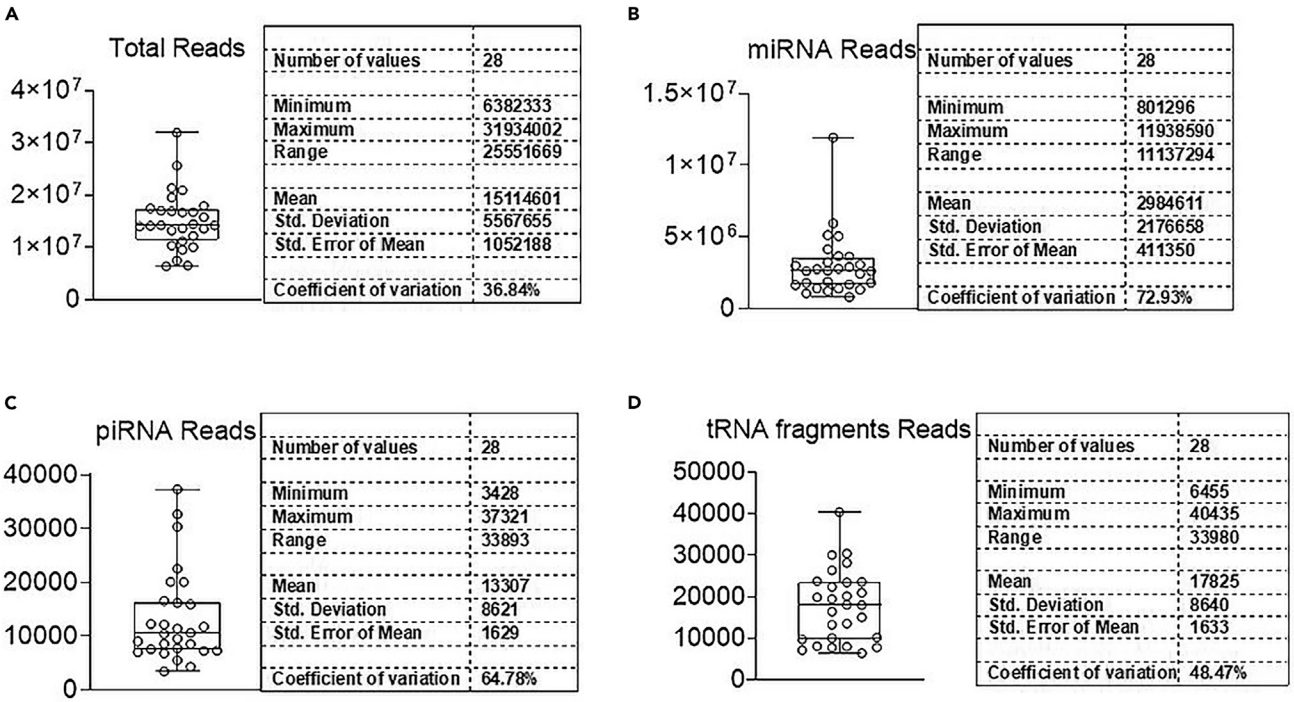

Figure 20. Box and whiskers plot show the reads distribution among samples and the descriptive statistics

Box and whiskers plot show the reads distribution among samples and the descriptive statistics (reported in the tables) of (A) Total reads, (B) miRNAs reads, (C) piRNAs reads and (D) tRNA fragments reads.

4. Finally, a normalization step is required since raw counts are not comparable between samples because of the library size (the total number of mapped reads) which typically varies from sample to sample.
5. Subsequently, 'EdgeR' R/Bioconductor package can be used to account for scaling the raw reads in counts per million (CPM) or the size factors can be estimated using the Median of Ratios method implemented in 'DESeq2' R/Bioconductor package EstimateSizeFactor function.

Clinical characteristics, raw UMI Reads, and CPM of miRNAs detected in the described dataset have been deposited on Mendeley data and are shown in Data S3 and can be used as a reference dataset for circulating miRNAs expression of healthy adult subjects. MiRNAs expression levels obtained by sequencing should be further validated using single assay qRT-PCR. To this aim, we suggest a data-driven approach based on the combination of three different algorithms as in Marabita et al. (Marabita et al., 2016) and Grieco et al. 2020 (Grieco et al., 2020) in order to establish candidate endogenous miRNAs to be exploited as reference normalizers. Each of the three algorithms generates a stability score such that a smaller score corresponds to higher expression stability combined into the summarized stability score (SSS) to select top candidate miRNAs in all three methods. Only miRNAs with complete observations has to be considered since we want to select miRNAs serving as endogenous controls, which should be abundantly and stably detected in all samples.

In more details:

6. To measure the stability of miRNAs expression subjects we used geNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004) which were implemented into the R/Bioconductor package NormqPCR (Perkins et al., 2012) and a CV-based score.

7. For each miRNA, geNorm calculates the pairwise variation (V) with all other miRNAs across the samples and defines a stability score (*M*) as the average V of a particular miRNA with all other control miRNAs.
8. Genes with the lowest M values have the most stable expression. Instead, Normfinder is a model-based approach which estimates the intergroup and intragroup variations of a miRNA, and then combines them into a stability value (*rho*).
9. Then, for each miRNA, calculate the coefficient of variation (CV) as its standard deviation across samples divided by the mean and scale it by the sum of all CVs for each sample (*CV score*). Lastly, calculate the SSS as the three-dimension Euclidean distance from the origin, i.e., $SSS = \sqrt{meanM^2 + rho^2 + CV\ score^2}$.

The analytical workflow was implemented in R language and is freely available as PCR Normalizator tool at the public repository https://github.com/romina/PCRNormalizator.

LIMITATIONS

The main limitations of the protocol could be the number of plasma samples that can be processed at a time in order to generate the cDNA libraries (n=8–16), and the quite long time required to complete a single prep (2 days).

TROUBLESHOOTING

Problem 1

Sometimes library amplification buffer could show white precipitates and cannot be used (Step 29a).

Potential solution

If you observe precipitates, continuously vortex for 2–3 min, briefly spin down and leave precipitates to dissolve at room temperature for 5 min.

Problem 2

During Libraries QC concentration evaluation using QUBIT spectrofluorometer, libraries with very low input could not be readable (step 31e).

Potential solution

If libraries are not readable, please consider to increase the amount of library to be evaluated from 1 μL to an upper volume of 5 μL.

Problem 3

Small amounts of total RNA can lead to a prevalence of adapters on small RNAs. It means that in QC analysis of your library/ies you will observe a prevalence of adapters peak (150–160 bp) (Figure 21). (step 32n).

Potential solution

In order to solve this problem, you do not necessarily have to increase RNA amount (for biofluids is very difficult), but you can scale the concentration of the adapters. For instance, if you used 3′ Adapters 1:5, scale to 1:10; and so, on 5′ Adapters 1:5 instead of 1:2.5 as well as RT primers 1:10 rather than 1:5. In this way, adapters and primers concentration will be proportional to the RNA amount, thus not prevailing on libraries generation.

Problem 4

Libraries dilution and normalization to 10 nM or 2 nM concentration is an essential step and could be difficult to reach the optimal final dilution, as measured using QUBIT spectrofluorometer (step 33, CRITICAL).

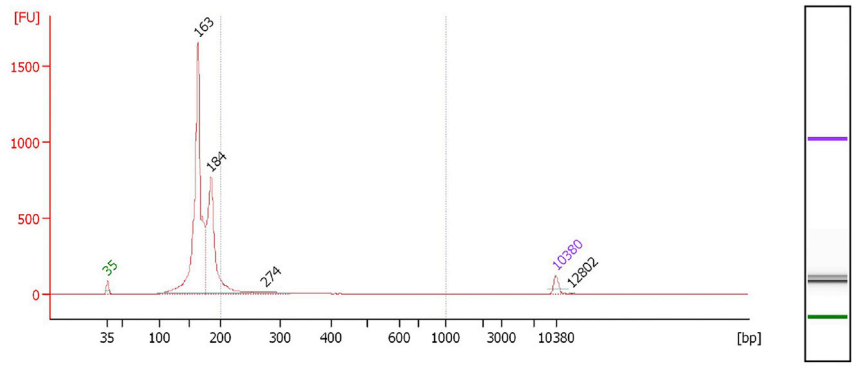

Figure 21. Electropherogram showing an example of small RNA library in which adapters prevail on small RNAs

Potential solution

Reagent contamination could be the primary source of such issue. Check all the reagents needed to normalize libraries concentration, including nuclease-free water.

Problem 5

In PCR validation step, if you are using a high number of Assays, it is possible to observe a cross-reaction among them. This cross-reaction is observed when qRT-PCR also amplifies negative control sample at the same Ct (or at a Ct very similar to those of samples) (Step 65, Important).

Potential solution

If cross-reaction is observed, look for sequence of other assays used. If an assay shows a very similar sequence to those analyzed that amplifies negative control sample, please consider elimination of at least one of these miRNAs (or to differently combine them), in order to avoid false results.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guido Sebastiani (sebastianiguido@gmail.com)

Materials availability

This study did not generate new unique reagents.

Data and code availability

Generated dataset are enclosed to the present manuscript. Original data of Data S1 and S2 *have been* deposited *to Mendeley data respectively:* https://doi.org/10.17632/7zcb24xbmy.1 *(https://data.mendeley.com/datasets/7zcb24xbmy/1)* and https://doi.org/10.17632/74msx7j24b.1 *(https://data.mendeley.com/datasets/74msx7j24b/1). These files contain Index nucleotide sequences to be used to create a new sample sheet onto Illumina Experiment Manager. Original data of Data S3 have been deposited to Mendeley data: https://doi.org/10.17632/vfj4yfffdd.1 (https://data.mendeley.com/datasets/vfj4yfffdd/1) The analytical workflow was implemented in R language and is freely available as PCR Normalizator tool at the public repository* https://github.com/romina/PCRNormalizator.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100606.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.S., F.D., C.G., and C.M.; methodology and validation, G.E.G., G.S., D.F., G.L., N.B., L.N., C.F., and R.D.; data curation, G.E.G., G.S., R.D., and M.B.; writing - original draft, G.E.G. and G.S.; writing, review, and editing, G.S., C.M., C.G., and F.D.; supervision, G.S., F.D., C.G., and C.M.; funding acquisition, F.D. and G.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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