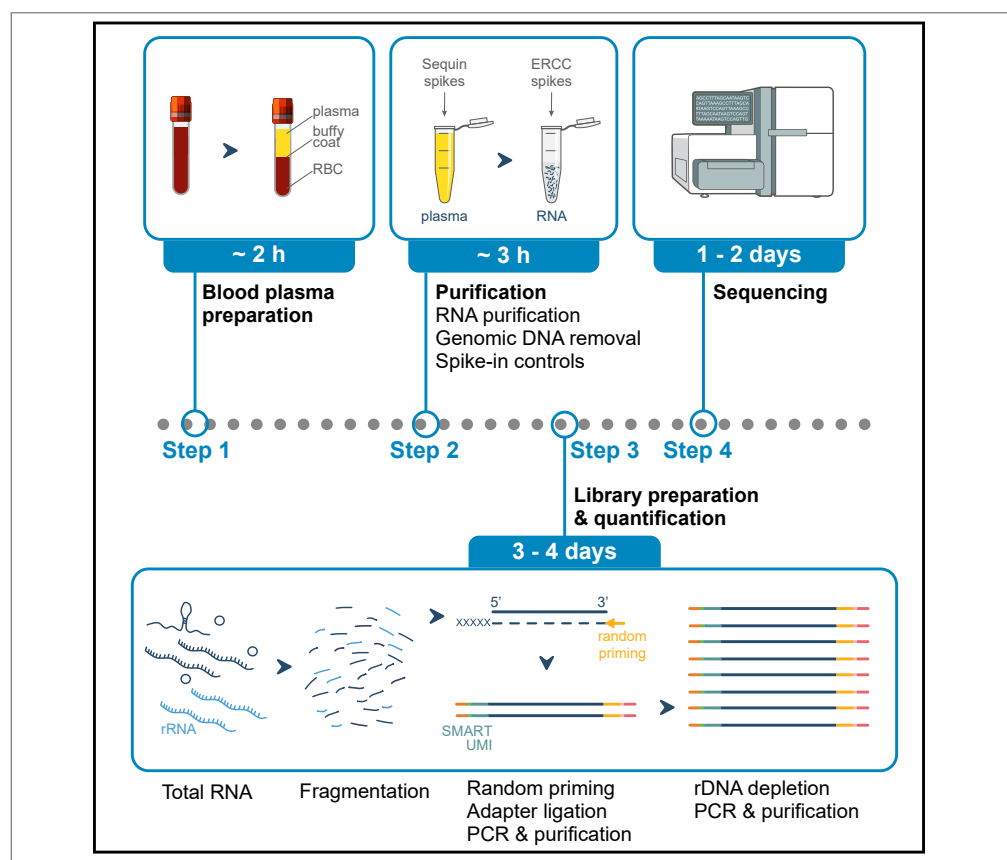


Protocol

Protocol for total RNA sequencing analysis of extracellular RNA from biofluids



Extracellular or cell-free RNAs (exRNAs) derived from biofluids are utilized in biomarker studies to study health and diseases. Here, we present a protocol for total RNA sequencing analysis of blood plasma exRNA using the Switching Mechanism At 5' end of RNA Template (SMART) cDNA synthesis technology. We describe all steps, from blood plasma preparation to sequencing data analysis. In addition to blood plasma, this protocol can also be applied to exRNA purified from other human, murine, and rat biofluids.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Anneleen Decock,
Kimberly Verniers,
Philippe
Decruyenaere,
Annelien Morlion,
Jasper Verwilt

annelien.morlion@ugent.
be

Highlights

Guidance on liquid
biopsy preparation
for extracellular RNA
(exRNA) profiling

Steps for library
preparation and
quantification for
total exRNA
sequencing

Templates to guide
experiment setup

Data analysis
instructions and
pipeline for total
exRNA sequencing

Decock et al., STAR Protocols
7, 104540
June 19, 2026 © 2026 The
Authors. Published by Elsevier
Inc.
[https://doi.org/10.1016/
j.xpro.2026.104540](https://doi.org/10.1016/j.xpro.2026.104540)



Protocol

Protocol for total RNA sequencing analysis of extracellular RNA from biofluids

Anneleen Decock,^{1,2} Kimberly Verniers,^{1,2} Philippe Decruyenaere,^{1,2,3} Annelien Morlion,^{1,2,6,7,8,*} and Jasper Verwilt^{4,5,6}

¹Department of Biomolecular Medicine, Ghent University, 9000 Ghent, Belgium

²OncoRNALab, Cancer Research Institute Ghent (CRIG), 9000 Ghent, Belgium

³Department of Hematology, Ghent University Hospital, 9000 Ghent, Belgium

⁴Department of Biomedical Sciences, University of Antwerp, 2000 Antwerp, Belgium

⁵Complex Genetics of Alzheimer's Disease Group, VIB Center for Molecular Neurology, 2000 Antwerp, Belgium

⁶These authors contributed equally

⁷Technical contact

⁸Lead contact

*Correspondence: annelien.morlion@ugent.be
<https://doi.org/10.1016/j.xpro.2026.104540>

SUMMARY

Extracellular or cell-free RNAs (exRNAs) derived from biofluids are utilized in biomarker studies to study health and diseases. Here, we present a protocol for total RNA sequencing analysis of blood plasma exRNA using the Switching Mechanism At 5' end of RNA Template (SMART) cDNA synthesis technology. We describe all steps, from blood plasma preparation to sequencing data analysis. In addition to blood plasma, this protocol can also be applied to exRNA purified from other human, murine, and rat biofluids.

For complete details on the use and execution of this protocol, please refer to Decruyenaere et al.¹

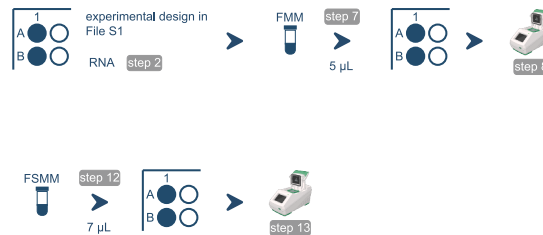
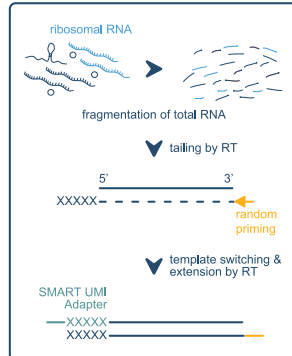
BEFORE YOU BEGIN

The protocol below describes the wet and dry lab steps for total RNA sequencing analysis of cell-free RNA (extracellular RNA, exRNA) purified from human, murine, or rat blood plasma, using the Switching Mechanism At 5' end of RNA Template (SMART) cDNA synthesis technology developed by Takara Bio.^{1–5} More specifically, we explain how to prepare and quantify strand-specific, ribosomal RNA (rRNA)-depleted sequencing libraries from DNase-treated exRNA, using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian, or a reconfigured version of this kit, i.e., the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit. Figure 1 summarizes the underlying principles of the library preparation, as well as the corresponding wet lab steps described in this section. This technology has also been used to profile exRNA purified from human urine³ and seminal plasma,⁶ from conditioned medium from cancer cells,³ and from blood plasma-, urine- and conditioned medium-derived extracellular vesicles.³

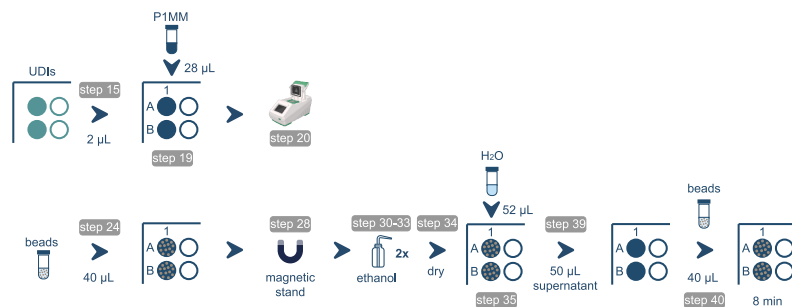
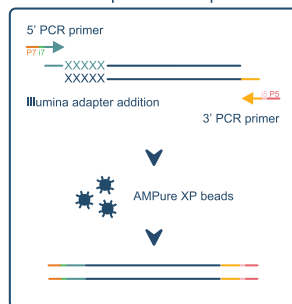
Note: The SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian and the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit are updated versions of the SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian (Cat#634411-634414 (with indexing primers) or Cat#634417-634419 (without indexing primers), Takara Bio). Also, this v2 version of the kit has been successfully applied to exRNA purified from liquid biopsies.^{3–6} Note that Takara Bio has communicated the phasing out of both the v2 and v3 versions of the kit. Therefore, we recommend to use the SMART-Seq Total RNA Pico Input



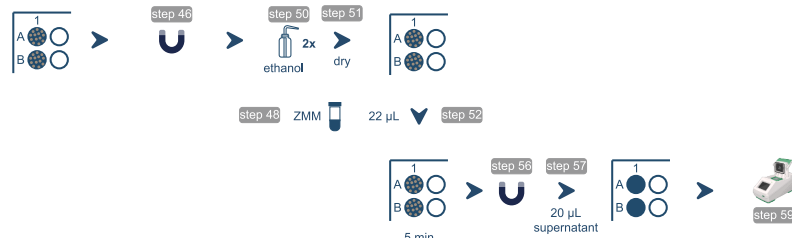
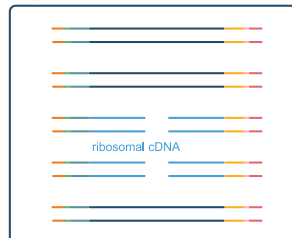
A 1st-strand cDNA synthesis



B 1st PCR amplification & purification



C depletion of ribosomal cDNA



D 2nd PCR amplification & purification

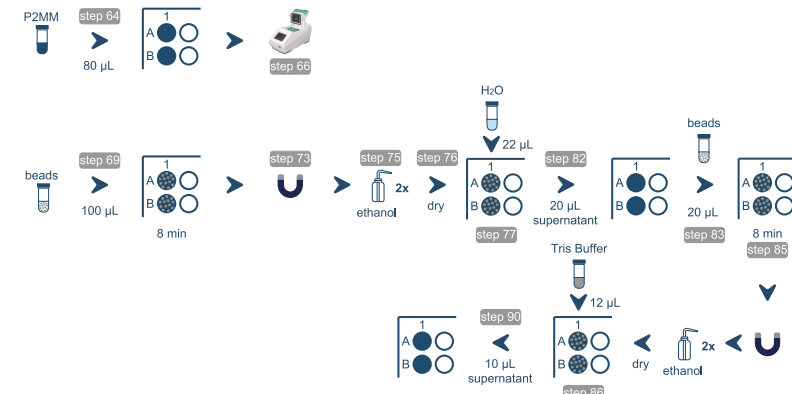
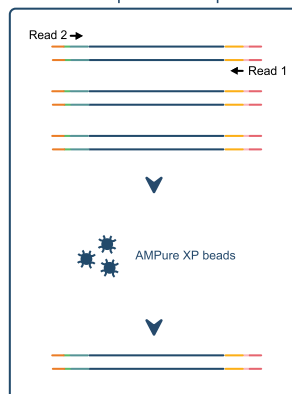


Figure 1. Schematic overview of the different steps in the total RNA sequencing library preparation workflow using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian or the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit

(A) The 1st-strand cDNA synthesis step starts with a DNase-treated, spike-in-supplemented RNA sample and involves total RNA fragmentation and cDNA synthesis. Random priming allows the generation of cDNA from all RNA fragments in the sample, including rRNA. When the SMARTScribe II Reverse Transcriptase reaches the 5' end of the RNA fragment, the enzyme's terminal transferase activity tails a few non-templated nucleotides to the 3' end of the cDNA (shown as XXXXX). The SMART UMI Adapter (included in the SMART UMI-TSO Mix v3) base-pairs with the non-templated nucleotide

Figure 1. Continued

stretch, initiating second-strand synthesis upon template switching, and contains 8 random nucleotides, which function as unique molecular identifiers (UMIs) in downstream analyses.

(B) In the next step, adapters for Illumina sequencing, including P5/P7 and i5/i7 indexes for flow cell clustering as well as regions recognized by sequencing primers for Read 1 and 2, are added via a first round of PCR amplification. The 5' PCR primer binds to the SMART UMI Adapter sequence. The 3' PCR primer binds to the sequence associated with the random primer.

(C) Ribosomal cDNA is then cleaved by ZapR Enzyme in the presence of mammalian-specific R-Probes. Non-ribosomal molecules are left untouched, with priming sites available on both 5' and 3' ends for further PCR amplification.

(D) The intact molecules are enriched by a second PCR amplification using P5/P7 primers, which are universal across all Illumina libraries. Read 1 generates sequences antisense to the original RNA, while Read 2 yields sequences sense to the original RNA. The first 8 nt of the second sequencing read (Read 2) are UMIs followed by 3 nucleotides of UMI-linker and 3 nontemplated nucleotides derived from the SMART UMI Adapter. Lastly, the PCR products are purified once more to yield the final cDNA library. The figure is based on the manufacturer's manuals and was partly created in BioRender (Mestdagh, P. (2026) <https://BioRender.com/qk3ojod>). FMM: Fragmentation Master Mix; FSMM: First-Strand Master Mix; P1MM: PCR1 Master Mix; ZMM: ZapR Master Mix; P2MM: PCR2 Master Mix.

with UMIs (ZapR Mammalian) kit for new studies. Furthermore, we recommend not to combine reagents from different kit versions, nor using different kit versions within a single experiment or study.

Innovation

This protocol is based on Takara Bio total RNA sequencing library preparation manuals (SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian and SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian)), with specific optimizations for exRNA isolated from liquid biopsies. Such RNA is typically present at very low concentrations and is highly fragmented, but profiling of these conditions is not fully addressed in existing protocols. We provide detailed guidance and quality control steps tailored to exRNA inputs to ensure robust and reproducible library preparation.

The protocol includes instructions for manual execution as proposed in the original manuals, as well as for semi-automated execution, enabling scalable, high-throughput processing while reducing operator-dependent variability. We also describe how the workflow can be further automated to support implementation in large studies.

To improve usability and reproducibility, we provide templates for experimental design and sequencing setup, as well as a user-friendly computational processing pipeline with test data. This end-to-end workflow enables users to run, evaluate, and adapt the protocol, guided by comprehensive quality control checks.

Institutional permissions

To collect human or animal biofluids, institutional regulations may legally require researchers to obtain ethical approval from the competent ethics committee of the host institution. Here, human blood plasma data of Decruyenaere et al.¹ were used, a study approved by the ethics committee of Ghent University Hospital (Ghent, Belgium; approval number EC/2016/0307). Written informed consent was obtained from all study participants.¹

Blood plasma preparation

⌚ Timing: ~2 h

⚠ CRITICAL: Note that different blood plasma preparation protocols may be used. However, pre-analytical variables highly impact the quantification of blood plasma-derived exRNAs.⁷ Therefore, we recommend standardizing blood sample collection and processing within a single study. Combining data from different blood collection tube types and/or different blood processing time intervals will introduce unacceptable biases into the results. We advise using citrate blood collection tubes and completing blood plasma preparation within 4 hours after collection.⁷ The use of manufacturer-designated preservation tubes (such as

the Cell-Free RNA BCT, Cell-Free DNA BCT, PAXgene Blood ccfDNA Tube, or Cell-Free DNA Collection Tube) should be avoided for exRNA profiling.⁷

Here, we describe the preparation of double-spun plasma from whole blood collected in Vacuette 9 mL, sodium citrate 3.2% blood collection tubes, following the International Society on Thrombosis and Haemostasis (ISTH) guidelines. The resulting plasma is directly suitable for exRNA profiling, but can also be used for extracellular vesicle purification and subsequent exRNA profiling.^{3,8}

1. Collect human venous blood from an elbow vein using a BD Vacutainer Push Button Blood Collection Set in Vacuette 9 mL, sodium citrate 3.2% blood collection tubes. Fill the blood collection tubes to the manufacturer's recommended volume.
2. Immediately after the blood draw, invert the blood collection tubes 5 times to mix the anticoagulant with the blood, then transport them at room temperature (18°C–22°C) to the lab for further processing.

Note: All centrifugation steps during the blood plasma preparation protocol are performed at room temperature (18°C–22°C) in a centrifuge with swinging bucket rotor and appropriate adapters, for example, a Centrifuge 5804 with rotor A-4-44 and adapters for blood collection tubes or 15 mL conical tubes. No or minimal acceleration and braking should be applied.

3. Before initiating the first centrifugation step, invert the blood collection tubes five times.
4. Prepare single-spun plasma:
 - a. Centrifuge the blood tubes at 2500 g for 15 min.
 - b. Carefully transfer the plasma using a 1 mL pipette to a 15 mL conical tube, leaving approximately 0.5 cm above the buffy coat. Do not touch the buffy coat with the pipette tip.

Note: Try not to disturb the buffy coat layer when preparing plasma. Carry-over of white blood cells and platelets from the buffy coat is the most likely source of cellular RNA contamination.

- c. Invert the 15 mL tube five times. Immediately continue with the next step.
5. Prepare double-spun plasma:
 - a. Centrifuge the 15 mL tube at 2500 g for 15 min.
 - b. Carefully transfer the plasma using a 1 mL pipette to a fresh 15 mL tube, leaving approximately 0.5 cm above the platelet pellet. Do not touch the pellet with the pipette tip.
 - c. Invert the 15 mL tube five times. Immediately continue with the next step.
6. Aliquot the double-spun plasma into 1.5 mL LoBind tubes, snap freeze in liquid nitrogen, and store at –80°C.

Note: To avoid freeze-thaw cycles and volume loss during pipetting, we recommend storing aliquots that can be used immediately as input volume for RNA purification (e.g., 200 µL plasma for the miRNeasy Serum/Plasma Kit).

Note: The number of centrifugation steps, and centrifugation speed and time impact the level of remaining blood platelets in the obtained plasma. ExRNA profiles from plasma with high platelet levels will be dominated by platelet RNA. To reduce the platelet RNA signal in the sequencing data, we recommend to centrifuge at least once at 2500 g for 15 min.⁴

Note: Using the aforementioned protocol, a completely filled blood collection tube yields approximately 3–5 mL of single-spun plasma, depending on the donor.

RNA purification and genomic DNA removal using spike-in RNA

⌚ Timing: ~3 h

Table 1. Sequin spike-in and External RNA Control Consortium (ERCC) spike-in RNA dilutions for total RNA sequencing of single-spun (SSP), double-spun (DSP), or triple-spun plasma (TSP)

Dilution name	Dilution relative to stock
Sequin stock ^a	
Sequin SSP ^b	1/250
Sequin DSP ^b	1/1500
Sequin TSP ^b	1/3000
ERCC stock ^a	
ERCC working solution	1/250
ERCC SSP ^b	1/5000
ERCC DSP ^b	1/12,500
ERCC TSP ^b	1/25,000

^aThe Sequin and ERCC spike-in RNA stock dilutions are a mixture of different RNA molecules at varying individual concentrations (see Supplemental information of Hulstaert et al.⁹). Note that only these individual RNA molecule concentrations are shared by the suppliers of these spike-in RNAs. Therefore, dilutions here are volume-based and not concentration-based. Dilutions are made using nuclease-free water.

^bSSP, DSP, and TSP are as prepared in the Extracellular RNA Quality Control study.⁷ Briefly, three successive centrifugation steps were performed, i.e., a first centrifugation step of 20 min at 400 g (resulting in SSP), followed by 10 min at 800 g (resulting in DSP), and a final centrifugation step of 15 min at 2500 g (resulting in TSP).

△ **CRITICAL:** Note that different RNA purification methods may be used to purify exRNA from blood plasma, considering compatibility with the selected downstream genomic DNA removal method.⁷ Keep in mind, however, that the choice of RNA purification method also impacts exRNA quantification.⁷ Therefore, we recommend standardizing processing methods within a single study. Combining data derived from different RNA purification methods will introduce unacceptable biases in the results. We advise to maximize the blood plasma input volume and recommend to use the miRNeasy Serum/Plasma Kit or miRNeasy Serum/Plasma Advanced Kit (for blood plasma input volumes of 0.2–0.6 mL), or the QIAamp ccfDNA/RNA Kit (for blood plasma input volumes up to 4 mL) for RNA purification.⁷

△ **CRITICAL:** Furthermore, the use of synthetic spike-in RNA during RNA purification^{8,10} and before genomic DNA (gDNA) removal is recommended to allow sequencing-based quality control (see Section [data analysis](#) in [expected outcomes](#)) and optional exRNA normalization.^{1–4,9,10} Depending on the applied centrifugation protocol to prepare plasma, different synthetic spike-in RNA dilutions are required. In [Table 1](#), we summarize the recommended Sequin (Garvan Institute of Medical Research)^{11,12} and External RNA Control Consortium (ERCC) spike-in RNA dilutions for single-spun, double-spun, and triple-spun plasma as prepared in the Extracellular RNA Quality Control Study.⁷

Here, we describe RNA purification using the miRNeasy Serum/Plasma Kit and gDNA removal using HL-dsDNase and Reaction buffer from 200 μ L plasma prepared as described in the Section [blood plasma preparation](#).

7. Follow the step-by-step details described in Hulstaert et al.,⁹ section RNA purification (step 1–11) and genomic DNA removal (step 12–21).
 - a. In step 2 of Hulstaert et al., use 2 μ L of Sequin TSP dilution ([Table 1](#)).

Note: The volume of Sequin dilution to be added to the lysate depends on the blood plasma input volume used for RNA purification. Per 100 μ L of plasma input, 1 μ L of the appropriate Sequin dilution ([Table 1](#)) is added to the sample lysate.

- b. In step 16 of Hulstaert et al., use 2 μ L of ERCC TSP dilution ([Table 1](#)).

Note: The volume of ERCC spike-in RNAs to be added to the RNA eluate depends on the eluate volume used for gDNA removal. Per 12 μ L of RNA eluate, 2 μ L of the appropriate ERCC dilution (Table 1) is added to the RNA sample.

Note: Sequin and ERCC spike-in RNA dilutions for profiling of human urine, conditioned medium from cancer cells, and extracellular vesicles, and for profiling of seminal plasma are indicated in Everaert et al.³ and Morlion et al.,⁶ respectively.

8. Store DNase-treated and spike-in-supplemented RNA eluates at -80°C . Avoid multiple freeze-thaw cycles.

Experimental design of the library preparation

⌚ Timing: ~15 min

The protocol described in the Section [step-by-step method details](#) is based on the manufacturer's manuals (SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian and SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) manuals) for the library preparation protocol of a single sample using monochannel pipettes. For profiling of multiple samples, the experimental template in [File S1](#) can be used to set up the library plate layout and to calculate the reagent volumes required to prepare the different master mixes throughout the protocol, as well as to determine how to aliquot these master mixes into the library plate, depending on the number of samples being profiled.

In addition, the Section [step-by-step method details](#) provides details for executing the protocol using (electronic) multichannel pipettes (see [key resources table](#) for complete list and matching pipetting tips) and/or semi-automated pipetting (using the VIAFLO 96 and Mantis Liquid Dispenser (with silicone high volume chips)) to accelerate profiling of large sample numbers. Although manual pipetting (using electronic multichannel pipettes) can be used to process up to 96 samples simultaneously, we recommend using semi-automated pipetting for sample numbers greater than 32. It should be noted that the semi-automated pipetting instructions require more reagents than manual pipetting does, due to allowances for dead volume. One must consider whether the benefits of faster library preparation outweigh the cost of increased reagent consumption. In the protocol steps, it is indicated which pipetting option(s) can be used: multichannel pipetting, Mantis, or VIAFLO 96. For the protocol steps that use the Mantis Liquid Dispenser, the time required to add the master mix to different sample numbers is summarized in [Table 2](#). For protocol steps in

Table 2. Dispense time of the Mantis Liquid Dispenser for each of the master mixes used in the library preparation protocol, depending on the number of samples being profiled

Sample number	5 μ L of FMM (min:s)	7 μ L of FSMM (min:s)	28 μ L of P1MM (min:s)	80 μ L of P2MM (min:s)
8	00:28	00:38	00:47	01:04
16	00:32	00:46	01:04	01:44
24	00:35	00:54	01:22	02:24
32	00:39	01:02	01:39	03:03
40	00:42	01:10	01:56	03:43
48	00:46	01:18	02:14	04:22
56	00:49	01:26	02:31	05:02
64	00:53	01:34	02:49	05:42
72	00:56	01:42	03:07	06:21
80	01:00	01:51	03:24	07:01
88	01:04	01:59	03:42	07:41
96	01:07	02:07	04:00	08:21

FMM, Fragmentation Master Mix; FSMM, First-Strand Master Mix; P1MM, PCR1 Master Mix; P2MM, PCR2 Master Mix.

which the VIAFLO 96 can be used, the appropriate electronic pipetting speed settings are specified in the protocol description.

Note: For all VIAFLO 96 steps, it is recommended to use the 'tip touch' technique: lightly touch the GripTips to the side of the well, or briefly dip them into the liquid after dispensing, to ensure complete liquid release and minimize carryover.

Unique dual-indexed PCR primers

⌚ Timing: ~10 min

To discriminate pooled libraries, unique dual-indexed PCR primers (UDIs) are used to generate indexed Illumina-compatible next-generation sequencing libraries (see Section [first-strand cDNA synthesis](#), step 15).

9. Select the correct UDIs, depending on the library preparation kit version used and the number of samples that need to be pooled.
 - a. Using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian:
 - i. For up to 24 samples: Use the SMARTer RNA Unique Dual Index Kit - 24U of the library preparation kit.
 - ii. More than 24 samples: Use the SMARTer RNA Unique Dual Index Kit - 96U Set A or B.

Note: The SMARTer RNA Unique Dual Index Kit - 96U Set A and B need to be ordered separately. More information on index sequences and pooling can be found in the [SMARTer RNA Unique Dual Index Kit Protocol-at-a-Glance documentation](#).

- b. Using the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian): Use the Unique Dual Index Kits.

Note: The Unique Dual Index Kits need to be ordered separately. More information on index sequences and pooling can be found in the [Unique Dual Index Kits Protocol-at-a-Glance documentation](#).

R-probes

⌚ Timing: ~10 min

During ribosomal cDNA depletion (see Section [depletion of ribosomal cDNA](#), step 44), library fragments originating from rRNA (18S and 28S) and mitochondrial rRNA (m12S and m16S) are cut by ZapR Enzyme in the presence of these R-Probes (mammalian-specific, included in both versions of the library preparation kit). These R-Probes target mammalian nuclear 28S, 18S, 5.8S, and 5S rRNA and human mitochondrial m16S and m12S rRNA sequences.

10. Aliquot the R-Probes at first use to limit the number of freeze-thaw cycles to 3 times.

Tris Buffer

⌚ Timing: ~10 min

During purification of the final RNA sequencing library, cDNA is eluted in Tris Buffer before library validation (see Section [purification of the final RNA sequencing library](#), step 86). This Tris Buffer is included in both versions of the library preparation kit.

11. Aliquot the Tris Buffer into 12 wells of tube strips.
12. Store the tube strips at 4 °C.

Note: Do not mix up this Tris Buffer with the 1 M Tris-HCl pH 8.0 needed to prepare the Library Quantification Dilution (LQD) Buffer and Pooling Buffer (see Section [materials and equipment](#) and [key resources table](#)).

KAPA Library Quantification Kit

⌚ Timing: ~1 h

Libraries are quantified using the KAPA Library Quantification Kit and the LightCycler480 (see Section [library quantification and pooling](#)). The KAPA kit contains DNA Standards 1–6, Primer Mix, and KAPA SYBR FAST qPCR Master Mix. Reagents should be prepared as follows.

13. Thaw the KAPA SYBR FAST qPCR Master Mix and Primer Mix at room temperature (18°C–22°C).

Note: The KAPA SYBR FAST qPCR Master Mix is light sensitive. Exposure to direct light for an extended period will result in a loss of fluorescent signal intensity. Protect the tube from light.

14. Vortex the Primer Mix tube and add 1 mL to the KAPA SYBR FAST qPCR Master Mix tube (5 mL) to obtain a total volume of 6 mL. Vortex the mixture.

Note: To minimize freeze-thaw cycles, 1 mL aliquots of this master mix can be made in 1.5 mL tubes.

15. Pipette the DNA Standards 1–6 (80 µL) into different wells of an 8-well PCR tube strip, as well as 80 µL PCR-grade water.

Note: This water will serve as a no-template control (NTC) sample for quantification. Volumes can be split across two tube strips to avoid freeze-thaw cycles.

16. Store master mix aliquots and DNA standards at –20 °C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Blood plasma	Human, murine, or rat donor	N/A
Chemicals, peptides, and recombinant proteins		
Ethanol, abs. 100%, HPLC grade	AnalytiChem Belgium NV	Cat#CL00.0529.1000
PCR-grade water	pxlence	Cat#PXL-PGW-1000
Invitrogen UltraPure Glycogen	Invitrogen	Cat#10757844
Sodium Acetate (3 M), pH 5.5, RNase-free	Invitrogen	Cat#AM9740
1 M Tris-HCl pH 8.0	Invitrogen	Cat#15568025
Tween20	Sigma-Aldrich	Cat#P1379
Critical commercial assays		
miRNeasy Serum/Plasma Kit	Qiagen	Cat#217184
miRNeasy Serum/Plasma Advanced Kit	Qiagen	Cat#217204
QIAamp ccfDNA/RNA Kit	Qiagen	Cat#55184
HL-dsDNase	ArcticZymes	Cat#70800-202

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Reaction buffer for gDNA removal	ArcticZymes	Cat#66001
SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian kit	Takara Bio	Cat#634485-634488
SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit	Takara Bio	Cat#634354-634356
SMARTer RNA Unique Dual Index Kit - 96U Set A (for use with the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian kit)	Takara Bio	Cat#634452
SMARTer RNA Unique Dual Index Kit - 96U Set B (for use with the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian kit)	Takara Bio	Cat#634457
Unique Dual Index Kits (for use with SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit)	Takara Bio	Cat#634752-634756
AMPure XP beads	Beckman Coulter Life Sciences	Cat#A63881
HS Small Fragment Kit	Agilent	Cat#DNF-477-0500
KAPA Library Quantification Kit	Roche	Cat#07960298001
1× dsDNA HS Assay Kit	ThermoFisher Scientific	Cat#33230
Pippin Gel Cassette - 2% Agarose, dye-free, w/internal standards, Pippin Prep, 100 – 600 bp	Sopachem	Cat#CDF2010
Deposited data		
Raw and analyzed data of Decruyenaere et al. ¹	The European Genome-Phenome Archive (EGA)	Dataset EGA: EGAD00001011679
Oligonucleotides		
Sequin spike-in RNA controls	Garvan Institute of Medical Research	https://sequins.bio/
ERCC RNA Spike-In Mix	ThermoFisher Scientific	Cat#4456740
Software and algorithms		
UMI-tools	Smith et al. ¹³	https://github.com/CGATOxford/UMI-tools
STAR	Dobin et al. ¹⁴	https://github.com/alexdobin/STAR
SAMtools	Danecek et al. ¹⁵	http://samtools.sourceforge.net
HTSeq	Anders et al. ¹⁶	https://github.com/htseq/htseq
RSeQC	Wang et al. ¹⁷	https://rseqc.sourceforge.net
FastQC	Andrews (available online) ¹⁸	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
R	R Core Team ¹⁹	https://www.r-project.org/
tidyverse	Wickham et al. ²⁰	https://tidyverse.org/
knitr	Xie Y et al. ²¹	https://cran.r-project.org/web/packages/knitr/index.html
plotly	Plotly Technologies Inc. ²²	https://plot.ly
Other		
BD Vacutainer Push Button Blood Collection Set	Becton Dickinson and Company	Cat#367326
Vacurette 9 mL, sodium citrate 3.2%	MLS	Cat#KG455322
Tube, 15 mL, PP, 17/120 mm, conical bottom	Greiner Bio-One International	Cat#188271
LoBind microcentrifuge tubes	Eppendorf	Cat#022431021
Centrifuge with swinging bucket rotor and appropriate adapters for blood collection tubes and 15 mL conical tubes	Eppendorf	Centrifuge 5804 (Cat#5804 000.013) with rotor A-4-44 and adapters 5804 753.003 (for blood collection tubes) and 5804 755.006 (for 15 mL conical tubes)
Cup Eppendorf Safe-Lock PCR Clean 1.5 mL and 2 mL	Eppendorf	Cat#A09000, A09001
Eppendorf Tube with Snap Cap 5.0 mL - Microtube	Eppendorf	Cat#15138344
Tubes, 0.2 mL, flat cap	ThermoFisher Scientific	Cat#AB0620
8 Well PCR Tube Strip, With Attached Caps	Azenta Life Sciences	Cat#4ti-0792
FrameStar 96 Well Non-Skirted PCR Plates, Clear Wells, Clear Frame	Azenta Life Sciences	Cat#4ti-0710/C

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Monochannel pipette P2, P10, P20, P100, P200 and P1000	Gilson	Cat#FA10001M - FA10006M
TripleA Filter Tips, Racked, Low Binding (tips for use with Gilson monochannel pipettes)	Westburg	Cat#WB 5010 L, WB 5020 L, WB 5030 L, WB 5040 L, WB 5074 L
Eppendorf Research plus, 12-channel, 10–100 μ L, yellow, variable volume (non-electronic)	Eppendorf	Cat#3125000044
Eppendorf Research plus, 12-channel, 30–300 μ L, orange, variable volume (non-electronic)	Eppendorf	Cat#3125000060
Dualfilter T.I.P.S. GLP, in rack, PCR clean, sterile (tips for use with Eppendorf multichannel pipettes)	Eppendorf	Cat#A40221- A40223
Pipet-Lite Multi Pipette L12-20XLS+ (non-electronic)	Mettler Toledo	Cat#17013808
E4 Multi Pipette Multi E12-20XLS+ (electronic)	Mettler Toledo	Cat#17013798
Filtertips ART 1–20 μ L low retention (tips for use with multichannel pipettes Cat#17013808 and Cat#17013798)	Rainin	Cat#A26102
Disposable reagent reservoir, 50 mL, sterile	Novolab	Cat#A06354
VIAFLO 96	Integra	Cat#6001, 6101, 6103, 6215, 6220
Integra GripTips - Low Retention, Sterile, Filter (tips for use with VIAFLO 96)	Integra	Cat#6555, 6435
150 mL Reagent Reservoir (reservoir for use with VIAFLO 96)	Integra	Cat#6301, 6302, 6337
Mantis Liquid Dispenser	Formulatrix	V3.3
Mantis Chip – Silicone, HV (1 μ L & 5 μ L), RFID, PI. Pack of 12	Formulatrix	Cat#233648
High-Speed Multi Plate Shaker	Biosan	Cat#BS-010216-A03, BS-010216-CK
T100 Thermal Cycler	Bio-Rad	Cat#186-1096
Adhesive PCR Plate Seals	ABgene	Cat#A00052
Cooling units	Greiner Bio-One	Cat#978201 (for 8-well PCR tube strips and PCR tubes of 0.2 mL) and 978203 (for 1 mL and 2 mL tubes)
Corning CoolRack XT PCR96, Holds 12 Strip Wells (cooling unit with SBS footprint for use with Mantis Liquid Dispenser and VIAFLO 96)	Corning	Cat#432053
Centrifuge for PCR plates	Eppendorf	Cat#5427 with rotor A-2-MTP
DynaMag-96 Side Magnet	Invitrogen	Cat#12331D
96-Well Side Pull Bar Magnet	Permagen Labware	Cat#MSP750
PCR Separation Plate		
Fragment Analyzer System	Agilent	Cat#M5310AA
FA 12-Capillary Array Short	Agilent	Cat#A2300-1250-3355
LightCycler480	Roche	Cat#05015243001
384-well PCR plates	ThermoFisher Scientific	Cat#12771206
Qubit 4 Fluorometer	ThermoFisher Scientific	Cat#Q33238
Pippin Prep System	Sage Science	Cat#PIP0001
Microcentrifuge	Novolab	Cat#A40681
Thermomixer	Novolab	Cat#A25796

MATERIALS AND EQUIPMENT

Storage conditions for library preparation reagents

Storage conditions for the library preparation reagents are summarized in [Table 3](#), along with instructions for thawing and storing them during library preparation.

Thermal cycler programs for library preparation

At multiple steps of the library preparation protocol, a T100 Thermal Cycler is used to incubate the samples according to different cycling protocols. We recommend pre-programming these cycling protocols ([Tables 4, 5, 6, 7, 8, and 9](#)) on the instrument before library preparation is started.

Table 3. Reagent storage conditions (during experiments)

Reagent	Storage (°C)	How to thaw	How to store during the experiment (upon thawing)
First-strand cDNA synthesis (day 1)			
SMART UMI-TSO Mix v3 ^a	−80 °C	Cooling unit	Cooling unit
SMART Pico Oligos Mix v3	−20 °C	Room temperature	Room temperature
5× First-Strand Buffer	−20 °C	Room temperature	Room temperature
SMARTScribe II Reverse Transcriptase ^b	−20 °C	Cooling unit	Cooling unit
RNase Inhibitor (40 U/μL) ^b	−20 °C	Cooling unit	Cooling unit
First PCR amplification (day 1)			
SeqAmp CB PCR Buffer (2×)	−20°C	Room temperature	Room temperature
PCR-grade water	4 or −20°C	Room temperature	Room temperature
Unique dual-indexed PCR primers	−20°C	Room temperature	Room temperature
SeqAMP DNA Polymerase ^b	−20°C	Cooling unit	Cooling unit
Purification of the RNA sequencing library (day 2)			
AMPure XP beads	4°C	Room temperature	Room temperature
Depletion of ribosomal cDNA (day 2)			
R-Probes ^{b,c}	−80°C	Cooling unit	Cooling unit
ZapR Buffer (10×)	−20°C	Room temperature	Room temperature
ZapR Enzyme ^{b,c}	−20°C	Cooling unit	Cooling unit
Second PCR amplification (day 2)			
SeqAmp CB PCR Buffer (2×)	−20°C	Room temperature	Room temperature
SeqAMP DNA Polymerase ^b	−20°C	Cooling unit	Cooling unit
PCR2 Primers v3	−20°C	Room temperature	Room temperature
Purification of the final RNA sequencing library (day 2 or day 3)			
Tris Buffer	4°C	Room temperature	Room temperature

Vortex and spin down all reagents before use.

Room temperature is defined as the range between 18°C and 22°C. Cooling unit refers to the range between −18°C and −20°C.

^aDo not vortex the SMART-UMI-TSO Mix v3.

^bDo not vortex the SMARTScribe II Reverse Transcriptase, RNase Inhibitor (40 U/μL), SeqAMP DNA Polymerase, R-Probes, and ZapR Enzyme, but flick the tubes and spin down.

^cNomenclature of reagents is according to the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit. In the v3 version of the kit, these products are indicated as R-Probes v3 and ZapR v3.

Sample dilution buffer for library quantification

Libraries are quantified using the KAPA Library Quantification Kit and the LightCycler480. Libraries need to be diluted using the buffer prepared in the overview below (see Section [library validation](#), step 97).

Library Quantification Dilution (LQD) Buffer

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8)	10 mM	500 μL
PCR-grade water	N/A	49.48 mL
Tween20	N/A	25 μL
Total	N/A	50 mL

Store at room temperature (18–22 °C) for up to 1 year.

Sample pooling buffer for library pooling

Libraries are equimolarly pooled upon library quantification (see Section [library quantification and pooling](#), steps 106 to 109). Libraries need to be pooled using Pooling Buffer, which can be prepared by combining 500 μL 1 M Tris-HCl (pH 8; final concentration is 10 mM) and 49.5 mL PCR-grade water to obtain a total volume of 50 mL. Store the Pooling Buffer at room temperature (18–22 °C) for up to 3 years.

STEP-BY-STEP METHOD DETAILS

⌚ **Timing:** The time required for each step in the protocol depends on the researcher's experience, the number of samples included in the library preparation, and whether semi-automated pipetting is used. For up to 96 samples, a skilled lab technician can complete the entire library preparation protocol in 3–4 days, depending on the chosen pipetting method. An additional day should be scheduled if the optional library size selection step is performed (see section [library size selection](#)). Note that the protocol includes defined safe stopping points; it cannot be paused at any other step. When performing the protocol for the first time, we recommend allowing sufficient time.

First-strand cDNA synthesis

⌚ **Timing:** Day 1

During this step, RNA is fragmented and first-strand cDNA synthesis is primed by the SMART Pico Oligos and uses the SMART UMI-TSO for template-switching at the 5' end of the transcript ([Figure 1A](#)).

Note: All steps of first-strand cDNA synthesis should be performed in a separate pre-PCR room to avoid contamination with PCR products.

1. Thaw the DNase-treated and spike-in-supplemented RNA eluate on a cooling unit. Flick the RNA eluate tube and spin down.
2. Pipette 8 μ L of RNA sample into a 96-well PCR plate on a cooling unit.
 - a. VIAFLO 96 option: transfer RNA samples in a 96-well PCR plate to a new 96-well PCR plate using the VIAFLO 96, with aspiration and dispense speeds set to 4.

Note: A cooling unit with SBS footprint is required for use of the VIAFLO 96. Classical cooling units are incompatible with the equipment.

3. Seal the plate and spin down.
4. Thaw the SMART UMI-TSO Mix v3 on a cooling unit (for use in step 12).
5. Thaw the SMART Pico Oligos Mix v3 and 5X First-Strand Buffer at room temperature (18°C–22°C).
6. Pre-heat a T100 Thermal Cycler according to the program indicated in [Table 4](#).
7. Prepare and add Fragmentation Master Mix (FMM; [File S1](#)).
 - a. Pipette 1 μ L of SMART Pico Oligos Mix v3 and 4 μ L 5 $\times$ First-Strand Buffer in a tube at room temperature (18°C–22°C).
 - b. Vortex the mixture and spin down.
 - c. Add 5 μ L of FMM to the RNA sample on a cooling unit (new sample volume: 13 μ L). Pipette up and down to mix.
 - i. Multichannel pipetting option: When using an electronic multichannel pipette, set aspiration and dispense speed to 3, mix speed to 5, and mix volume to 11 μ L.

Table 4. Protocol steps 6 and 8: Thermal cycler program to fragment RNA with lid temperature set to 105 °C and volume set to 13 μ L

Steps	Temperature	Time	Cycles
Pre-heat	94 °C	∞	1 cycle
Incubation	94 °C	2 min	1 cycle
Hold	12 °C	∞	1 cycle

Estimated time: $\pm$ 3 min.

Table 5. Protocol steps 11 and 13: Thermal cycler program to synthesize first-strand cDNA with lid temperature set to 105 °C and volume set to 20 µL

Steps	Temperature	Time	Cycles
Pre-heat	42°C	∞	1 cycle
Incubation	42°C	90 or 180 min ^a	1 cycle
Incubation	70°C	10 min	1 cycle
Hold	12°C	∞	1 cycle

Estimated time: ± 100 or 190 min.

^aWhen using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian, this incubation time is set to 180 min. When using the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit, this incubation time is set to 90 min.

- ii. Mantis option: Evaluate the time needed to add the FMM based on [Table 2](#).

Note: A cooling unit with an SBS footprint is required for use of the Mantis Liquid Dispenser. Classical cooling units are incompatible with the equipment.

- d. Seal the plate, spin down, and verify that there are no air bubbles.
8. Place the 96-well PCR plate on a pre-programmed T100 Thermal Cycler and incubate according to the program described in [Table 4](#).
9. Meanwhile, place the SMARTscribe II Reverse Transcriptase and RNase Inhibitor on a cooling unit (for use in step 12).
10. Ideally, place the plate from the thermal cycler on a cooling unit for 2 min once the second step in [Table 4](#) is completed. At the latest, move the plate from the thermal cycler to a cooling unit for 2 min when the thermal cycler reaches 12°C.
11. Pre-heat a thermal cycler according to the program indicated in [Table 5](#). Select the appropriate incubation time, depending on the library preparation kit version that is used.
12. Prepare and add First-Strand Master Mix (FSMM; [File S1](#)).
 - a. Pipette 4.5 µL of SMART UMI-TSO Mix v3, 0.5 µL of RNase inhibitor, and 2 µL of SMARTscribe II Reverse Transcriptase into a tube on a cooling unit.

Note: The SMART UMI-TSO Mix v3 is very viscous. Therefore, slowly pipette all components into the mixture. Only when all components have been added, pipette up and down 10 times to homogenize the FSMM.

- b. Spin down the 96-well PCR plate.
- c. Add 7 µL of the FSMM to the sample well (new sample volume: 20 µL). Pipette up and down to mix.
 - i. Multichannel pipetting option: When using an electronic multichannel pipette, set aspiration and dispense speed to 2, mix speed to 6, and mix volume to 17 µL.
 - ii. Mantis option: Evaluate the time needed to add the FSMM based on [Table 2](#).
- d. Seal the plate and spin down.
13. Place the 96-well PCR plate on a pre-programmed thermal cycler and incubate according to the program described in [Table 5](#).

Note: Leave the plate in the thermal cycler at 12°C until the next step.

Note: Meanwhile, thaw the unique dual-indexed PCR primers (UDIs) and SeqAmp CB PCR Buffer (2×) at room temperature (18–22 °C; for use in [Section first PCR amplification](#), step 15 and 19, respectively). The SeqAmp CB PCR Buffer (2×) may remain in a liquid state at –20 °C. In that case, place the buffer at 4 °C until the UDIs are thawed. Then, place the tube at room temperature (18–22 °C) to equilibrate the buffer for 10–20 min.

Table 6. Protocol steps 18 and 20: Thermal cycler program for the first RNA sequencing library amplification by PCR with lid temperature set to 105 °C and volume set to 50 μ L

Steps	Temperature	Time	Cycles
Pre-heat	94°C	∞	1 cycle
Incubation	94°C	1 min	1 cycle
Denaturation	98°C	15 s	5 cycles
Annealing	55°C	15 s	
Extension	68°C	30 s	
Incubation	68°C	2 min	1 cycle
Hold	12°C	∞	1 cycle

Estimated time: $\pm$ 15 min.

First PCR amplification

⌚ Timing: Day 1

The first-strand cDNA is amplified into RNA sequencing libraries using SeqAmp DNA Polymerase, and Illumina adapters and indexes are incorporated via the UDIs from the Unique Dual Index Kits (Figure 1B).

Note: All steps prior to PCR amplification on the thermal cycler (step 20) should be performed in a separate pre-PCR room to prevent contamination with PCR products.

14. Upon incubation in the thermal cycler, spin down the 96-well PCR plate.
15. Add 2 μ L of the appropriate UDI to each sample well (new sample volume: 22 μ L).

Note: Since the UDIs are provided in 96-well plates by Takara Bio, they can be added using multichannel pipetting or using the VIAFLO96 with aspiration and dispense speeds set to 4.

16. Seal the plate and spin down.
17. Place the SeqAmp DNA Polymerase on a cooling unit.
18. Pre-heat a T100 Thermal Cycler according to the program indicated in Table 6.
19. Prepare and add PCR1 Master mix (P1MM; File S1).
 - a. Pipette 2 μ L of PCR-grade water, 25 μ L of SeqAmp CB PCR Buffer (2X), and 1 μ L of SeqAmp DNA Polymerase into a tube on a cooling unit.
 - b. Pipette up and down to homogenize the mixture and spin down.
 - c. Add 28 μ L of P1MM to each sample well on a cooling unit (new sample volume: 50 μ L). Pipette up and down to mix.
 - i. Multichannel pipetting option: Use a multichannel pipette to add the P1MM to the library preparation plate.
 - ii. Mantis option: Evaluate the time needed to add the P1MM based on Table 2.
 - d. Seal the plate and spin down.
20. Place the 96-well PCR plate on a pre-programmed thermal cycler and incubate according to the program described in Table 6.

⏸ Pause point: The library preparation protocol can be paused here safely. Seal the plate and store at -20°C for up to 2 weeks.

Purification of the RNA sequencing library

⌚ Timing: Day 2

The amplified RNA sequencing library is purified by immobilizing it onto AMPure XP beads. The beads are then washed with 80% ethanol, and the cDNA is eluted with PCR-grade water before ribosomal cDNA depletion ([Figure 1B](#)).

Note: Purification of the RNA sequencing library should be performed in a post-PCR room.

21. Bring AMPure XP beads to room temperature (18–22 °C) for at least 30 min.
22. Thaw the 96-well PCR plate at room temperature (18–22 °C) and spin down.
23. Vortex the AMPure XP beads until they are well dispersed.
24. Add 40 µL of well-dispersed AMPure XP beads to the sample well (new sample volume: 90 µL).
 - a. Multichannel pipetting option:
 - i. Pour the beads into a disposable reagent reservoir.
 - ii. Add 40 µL of beads to the library preparation plate.
 - b. VIAFLO 96 option:
 - i. Pour the beads into a VIAFLO 96-compatible reagent reservoir.
 - ii. Add 40 µL of beads to the library preparation plate using the VIAFLO 96, with aspiration and dispense speeds set to 1.
25. Seal the plate and pulse vortex for 5 s.
26. Incubate the plate at room temperature (18–22°C) for 8 min to let the DNA bind to the beads.

Note: Meanwhile, also prepare two fresh 96-well PCR plates with well-dispersed AMPure XP beads when multichannel pipetting or the VIAFLO 96 was used in step 24. The first plate is for use in step 39 and should contain 40 µL beads in each sample well. The second plate is for use in Section [purification of the final RNA sequencing library](#), step 82 and should contain 20 µL beads in each sample well. Seal the plates and store at 4 °C.

Note: Meanwhile, thaw the R-Probes on a cooling unit (for use in Section [depletion of ribosomal cDNA](#), step 44), thaw the ZapR Buffer at room temperature (18–22°C; for use in Section [depletion of ribosomal cDNA](#), step 48), and bring PCR-grade water to room temperature (18–22 °C).

27. Spin down the plate for 1 min at 280 g.
28. Place the plate on a magnetic stand at room temperature (18–22 °C) for 5 min or longer, until the solution is completely clear.

Note: When using the VIAFLO 96 for subsequent bead clean-ups (i.e., steps 30, 35, Section [depletion of ribosomal cDNA](#), step 50, and Section [purification of the final RNA sequencing library](#), step 75,77 and 85), make sure to use a VIAFLO 96 compatible magnetic stand from step 28 onwards, for example, the 96-Well Side Pull Bar Magnet PCR Separation. Do not transfer the library preparation plate from one magnetic stand to another.

29. While the plate is on the magnetic stand, remove 80 µL of supernatant without disturbing the beads.

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out (see Section [problem 1](#) and [potential solution in troubleshooting](#)).

30. Keep the plate on the magnetic stand. Add 200 µL of freshly prepared 80% ethanol to each sample well, without disturbing the beads. Wait 30 s, then carefully remove the supernatant.
 - a. Multichannel pipetting option:
 - i. Pour the ethanol into a disposable reagent reservoir.
 - ii. Add 200 µL of ethanol to the library preparation plate.
 - iii. Remove the supernatant.

b. VIAFLO 96 option:

- i. Pour the ethanol into a VIAFLO 96-compatible reagent reservoir.
- ii. Add 200 μ L of ethanol to the library preparation plate using the VIAFLO 96, with aspiration speed 10 and dispense speed 7.
- iii. Remove the supernatant, using aspiration speed 3 and dispense speed 7.

Note: Make sure that no beads are pipetted out.

31. Repeat step 30 once.

32. Seal the plate and spin it for 1 min at 280 g to collect the remaining ethanol at the bottom of each tube.

33. Place the plate at the magnetic stand for 30 s. Remove all the remaining ethanol.

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out.

34. Keep the plate on the magnetic stand. Let the plate rest open at room temperature (18–22 °C) for 3–5 min until the pellet appears dry. Check the pellet frequently and proceed to the next step once the beads are dry.

Note: If the pellet is overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads, resulting in reduced recovery.

35. Keep the plate on the magnetic stand. Add 52 μ L of PCR-grade water to cover the beads (new sample volume: 52 μ L).

a. Multichannel pipetting option:

- i. Pour PCR-grade water into a disposable reagent reservoir.
- ii. Add 52 μ L of water to the library preparation plate.

b. VIAFLO 96 option:

- i. Pour PCR-grade water into a VIAFLO 96-compatible reagent reservoir.
- ii. Add 52 μ L of water to the library preparation plate using the VIAFLO 96, with aspiration speed set to 3 and dispense speed set to 7.

36. Seal the plate, remove it from the magnetic stand, and flick the wells until the beads are resuspended.

37. Incubate the plate at room temperature (18–22°C) for 5 min to rehydrate.

38. Spin down the plate, remove the seal, and place it on the magnetic stand for 2 min or longer, until the solution is completely clear.

39. Keep the plate on the magnetic stand. Transfer 50 μ L of clear supernatant containing the purified RNA sequencing library to a fresh 96-well PCR plate (see Section [problem 1](#) and [potential solution](#) in [troubleshooting](#); new sample volume: 50 μ L).

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out.

Note: When a fresh 96-well PCR plate with beads was prepared in step 24, transfer the clear supernatant directly to this plate, and skip step 40.

40. Add 40 μ L well-dispersed AMPure XP beads to the sample well (new sample volume: 90 μ L).

a. Multichannel pipetting option:

- i. Pour the beads into a disposable reagent reservoir.
- ii. Add 40 μ L of beads to the library preparation plate.

b. VIAFLO 96 option:

- i. Pour the beads into a VIAFLO 96-compatible reagent reservoir.

Table 7. Protocol steps 43 and 44: Thermal cycler program for pre-heating the R-Probes with lid temperature set to 105°C and volume set to the corresponding volume of the R-Probes used

Steps	Temperature	Time	Cycles
Pre-heat	72°C	∞	1 cycle
Incubation	72°C	2 min	1 cycle
Hold	12°C	∞	1 cycle

Estimated time: ± 3 min.

- ii. Add 40 µL of beads to the library preparation plate using the VIAFLO 96, with aspiration and dispense speed set to 1.
41. Seal the plate and pulse vortex for 5 s.
42. Incubate the plate at room temperature (18–22°C) for 8 min to let the DNA bind to the beads.

Depletion of ribosomal cDNA

⌚ Timing: Day 2

Library fragments originating from rRNA (18S and 28S) and mitochondrial rRNA (m12S and m16S) are cut by ZapR Enzyme in the presence of R-Probes (mammalian-specific). These R-Probes target mammalian nuclear 28S, 18S, 5.8S, and 5S rRNA and human mitochondrial m16S and m12S rRNA sequences. Non-human mitochondrial rRNA sequences will not be removed (Figure 1C).

Note: Steps 43–44 and steps 47–48 should be performed in a separate, pre-PCR room to avoid contamination with PCR products. All other steps should be performed in a post-PCR room.

43. Pre-heat a thermal cycler according to the program indicated in Table 7.
44. Prepare R-Probes.
 - a. Pipette a sufficient volume of R-Probes for the number of reactions to be performed into a PCR tube on a cooling unit. Use 1.5 µL per reaction, and add an extra 1 to 2 µL to the total volume to ensure sufficient volume for transfer in step 48.
 - b. Spin down, place the tube into the pre-programmed thermal cycler, and incubate according to the program described in Table 7.

Note: Leave the R-Probes in the thermal cycler at 12°C for at least 2 min, but for no more than 10–15 min, before using it to make the ZapR Master Mix (step 48).

Note: Immediately return the remaining unused R-Probes to –80°C for storage.

45. Upon incubation (step 42), spin the 96-well PCR plate at 280 g for 1 min.
46. Place the plate on a magnetic stand at room temperature (18–22 °C) for 5 min or longer, until the solution is completely clear.
47. Place ZapR Enzyme on a cooling unit.
48. Prepare ZapR Master Mix (ZMM; File S1). Select option a or b, depending on the library preparation kit version used.
 - a. Using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian: Pipette 16.80 µL of PCR-grade water, 2.20 µL of 10× ZapR Buffer, 1.5 µL of ZapR v3, and 1.5 µL of R-Probes v3 in a tube at room temperature (18–22°C).
 - b. Using the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit: Pipette 15.50 µL of PCR-grade water, 2.20 µL of 10× ZapR Buffer, 2.80 µL of ZapR Enzyme, and 1.50 µL of R-Probes in a tube at room temperature (18–22°C).

Table 8. Protocol steps 55 and 59: Thermal cycler program for depletion of ribosomal cDNA with lid temperature set to 105 °C and volume set to 20 µL

Steps	Temperature	Time	Cycles
Pre-heat	37°C	∞	1 cycle
Incubation	37°C	60 min	1 cycle
Incubation	72°C	10 min	1 cycle
Hold	12°C	∞	1 cycle

Estimated time: ± 70 min.

Note: The ZapR Enzyme is very viscous. Therefore, slowly pipette all components into the mixture in the same order as listed above. Ensure that the preheated and chilled R-Probes are added last. Only when all ZMM components have been added, pipette up and down 10 times to homogenize the mix and spin down.

Note: Immediately return the ZapR Enzyme to –20°C after use.

Note: When using the multichannel option to add the ZMM to the library preparation plate in step 52, aliquot the ZMM into strip(s).

49. While the plate is on the magnetic stand, remove 80 µL of supernatant without disturbing the beads.

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out (see Section [problem 1](#) and [potential solution](#) in [troubleshooting](#)).

50. Repeat Section [purification of the RNA sequencing library](#), step 30 to 33.
51. Keep the plate on the magnetic stand. Let the plate rest open at room temperature (18–22°C) for 1–2 min until the pellet appears dry. Check the pellet frequently and proceed to the next step once the beads are dry.

Note: The beads will dry more quickly than in Section [purification of the RNA sequencing library](#), step 34.

52. Keep the plate on the magnetic stand. Pipette 22 µL of the ZMM onto the beads (new sample volume: 22 µL).
 - a. Multichannel pipetting option: Use the strip prepared in step 48 to add ZMM to the library preparation plate.
53. Seal the plate, remove it from the magnetic stand, and flick the wells until the beads are resuspended.
54. Incubate the plate at room temperature (18–22°C) for 5 min to rehydrate.
55. Pre-heat a thermal cycler according to the program indicated in [Table 8](#).
56. Spin down the plate, remove the seal, and place it on the magnetic stand for 2 min or longer, until the solution is completely clear.
57. Keep the plate on the magnetic stand. Transfer 20 µL of clear supernatant to a fresh 96-well PCR plate without disturbing the beads (new sample volume: 20 µL).

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out (see Section [problem 1](#) and [potential solution](#) in [troubleshooting](#)).

58. Seal the fresh 96-well PCR plate and spin down at 280 g for 1 min.
59. Place the 96-well PCR plate on the pre-programmed thermal cycler and incubate according to the program described in [Table 8](#).

Table 9. Protocol steps 61 and 66: Thermal cycler program for the second RNA sequencing library amplification by PCR with lid temperature set to 105 °C and volume set to 100 µL

Steps	Temperature	Time	Cycles
Pre-heat	94°C	∞	1 cycle
Incubation	94°C	1 min	1 cycle
Denaturation	98°C	15 s	16 cycles
Annealing	55°C	15 s	
Extension	68°C	30 s	
Hold	12°C	∞	1 cycle

Estimated time: ± 35 min.

Second PCR amplification

⌚ Timing: Day 2

Library fragments not cleaved during the ZapR reaction are enriched in a second round of PCR. Since barcodes have already been added to the libraries, a single pair of primers can be used (Figure 1D).

Note: Steps 60 and 62 should be performed in a separate, pre-PCR-amplification room to avoid contamination with PCR products. All other steps should be performed in a post-PCR room.

60. Bring the PCR2 Primers v3, PCR-grade water, and SeqAmp CB PCR Buffer (2×) to room temperature (18–22°C). Place the SeqAmp DNA Polymerase on a cooling unit.
61. Pre-heat a thermal cycler according to the program indicated in Table 9.
62. Prepare the PCR2 Master Mix (P2MM; File S1).
 - a. Pipette 26 µL of PCR-grade water, 50 µL of SeqAmp CB PCR Buffer, 2 µL of PCR2 Primers v3, and 2 µL of SeqAmp DNA Polymerase in a tube on a cooling unit (total volume: 80 µL).
 - b. Pipette up and down to homogenize the P2MM and spin down.

Note: For P2MM volumes exceeding 2000 µL (i.e., more than 25 samples), vortex it rather than pipetting up and down to ensure homogenization.

Note: When using the multichannel option to add the P2MM to the library preparation plate in step 64, aliquot the P2MM into strip(s).

63. Remove the 96-well PCR plate from the thermal cycler, spin down, and keep the plate on a cooling unit.
64. Add 80 µL of P2MM to each sample well on a cooling unit (new sample volume: 100 µL). Pipette up and down to homogenize.
 - a. Multichannel pipetting option: Use the strip(s) prepared in step 62 to add P2MM to the library preparation plate.
 - b. Mantis option: Evaluate the time needed to add the P2MM based on Table 2.
65. Seal the plate and spin down.
66. Place the 96-well PCR plate on the pre-programmed thermal cycler and incubate according to the program described in Table 9.

Note: Upon starting the incubation on the thermal cycler, evaluate whether the next step (i.e., Purification of the final RNA sequencing library) can be completed on the same day (depending on the pipetting method that is chosen). When the protocol is continued on the same day, allow the AMPure XP beads to reach room temperature (18–22°C) before use in the purification of the final RNA sequencing library.

▮▮ Pause point: The library preparation protocol can be safely paused here. Seal the plate and store at -20°C for up to 2 weeks.

Purification of the final RNA sequencing library

⌚ Timing: When semi-automated pipetting is used, this step can be performed on day 2. When no semi-automated pipetting is used, this step should be initiated at day 3. In that case, bring the AMPure XP beads to room temperature (18°C – 22°C) before use.

The amplified RNA sequencing library is purified by immobilizing it onto AMPure XP beads. The beads are then washed and the cDNA is eluted before library validation ([Figure 1D](#)).

67. Spin down the 96-well PCR plate at 280 g for 1 min.
68. Vortex the AMPure XP beads until they are well dispersed.
69. Add 100 μL of well-dispersed AMPure XP beads to the sample well (new sample volume: 200 μL).
 - a. Multichannel pipetting option:
 - i. Pour the beads into a disposable reagent reservoir.
 - ii. Add 100 μL of beads to the library preparation plate.
 - b. VIAFLO 96 option:
 - i. Pour the beads into a VIAFLO 96-compatible reagent reservoir.
 - ii. Add 100 μL of beads to the library preparation plate using the VIAFLO 96, with aspiration and dispense speeds set to 1.
70. Seal the plate and pulse vortex for 5 s.
71. Incubate the plate at room temperature (18 – 22°C) for 8 min to let the DNA bind to the beads.
72. Spin down the plate for 1 min at 280 g.
73. Place the plate on a magnetic stand at room temperature (18 – 22°C) for 5–10 min or longer, until the solution is completely clear.
74. While the plate is on the magnetic stand, remove 190 μL of supernatant without disturbing the beads.

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out (see [Section problem 1](#) and [potential solution in troubleshooting](#)).

75. Repeat [Section purification of the RNA sequencing library](#), step 30 to 33.
76. Keep the plate on the magnetic stand. Let the plate rest open at room temperature (18 – 22°C) for 3–7 min until the pellet appears dry. Check the pellet frequently and continue to the following step when the beads are dry.

Note: If the pellet is overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads, resulting in reduced recovery.

77. Keep the plate on the magnetic stand. Add 22 μL of PCR-grade water to cover the beads (new sample volume: 22 μL).
 - a. Multichannel pipetting option:
 - i. Pour PCR-grade water into a disposable reagent reservoir.
 - ii. Add 22 μL of water to the library preparation plate.
 - b. VIAFLO 96 option:
 - i. Pour PCR-grade water into a VIAFLO 96-compatible reagent reservoir.
 - ii. Add 22 of μL water to the library preparation plate using the VIAFLO 96, with aspiration speed 3 and dispense speed 7.
78. Seal the plate, remove it from the magnetic stand, and flick the wells until the beads are resuspended.
79. Incubate the plate at room temperature (18 – 22°C) for 5 min to rehydrate.

80. Meanwhile, equilibrate the Tris Buffer of the library preparation kit at room temperature (18–22 °C; for use in step 86).
81. Spin down the plate, remove the seal, and place it on the magnetic stand for 2 min or longer, until the solution is completely clear.
82. Keep the plate on the magnetic stand. Transfer 20 μ L of clear supernatant containing the purified RNA sequencing library to a fresh 96-well PCR plate (new sample volume: 20 μ L).

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out (see Section [problem 1](#) and [potential solution](#) in [troubleshooting](#)).

Note: When a fresh 96-well PCR plate with beads was prepared in Section [purification of the RNA sequencing library](#), step 24, transfer the clear supernatant directly to this plate, and skip step 83.

83. Add 20 μ L well-dispersed AMPure XP beads to the sample well (new sample volume: 40 μ L).
 - a. Multichannel pipetting option:
 - i. Pour the beads into a disposable reagent reservoir.
 - ii. Add them to the library preparation plate.
 - b. VIAFLO 96 option:
 - i. Pour the beads into a VIAFLO 96-compatible reagent reservoir. Add them to the library preparation plate using the VIAFLO 96, with aspiration and dispense speeds set to 1.
84. Seal the plate and pulse vortex for 5 s.
85. Repeat steps 71–76 to perform a second round of bead clean-up.

Note: The beads will dry more quickly than in step 76.

86. Once the beads are dry, pipette 12 μ L of Tris Buffer from the library preparation kit onto the beads (new sample volume: 12 μ L).
 - a. Multichannel pipetting option: Use Tris Buffer aliquoted into strips (see Section [Tris Buffer](#) in [before you begin](#)).

Note: Depending on the number of quality checks and the sequencing volume required, consider using 20 μ L instead of 12 μ L. In that case, transfer 18 μ L in step 90.

87. Seal the plate, remove it from the magnetic stand, and flick the wells until the beads are resuspended.
88. Incubate the plate at room temperature (18–22 °C) for 5 min to rehydrate.
89. Spin down the plate, remove the seal, and place it on the magnetic stand for 2 min or longer, until the solution is completely clear.
90. Keep the plate on the magnetic stand. Transfer 10 μ L of clear supernatant to a fresh 96-well PCR plate without disturbing the beads (new sample volume: 10 μ L).

Note: This step can also be performed using multichannel pipetting. Make sure that no beads are pipetted out (see Section [problem 1](#) and [potential solution](#) in [troubleshooting](#)).

⏸ Pause point: The library preparation protocol can be paused here safely. Seal the plate and store at –20 °C.

Library validation

⌚ **Timing:** Day after purification of the final RNA sequencing library (i.e., day 3 or day 4)

Libraries are validated using the Fragment Analyzer System, FA 12-Capillary Array Short, and HS Small Fragment Kit. Library size distribution and average size are determined using the ProSize Fragment Analyzer data analysis software.

91. Use 1 μ L of the library obtained in Section [purification of the final RNA sequencing library](#), step 90 and add 1 μ L of PCR-grade water.
92. Load 2 μ L of diluted library onto the Fragment Analyzer and run the samples according to the [manufacturer's instructions](#).
93. Check the size and purity of the sample.

Note: The final product should be a smear from 200 bp to 1000 bp with a peak at approximately 300 bp (see [Figure 2](#) in Section [expected outcomes](#), and [problem 2](#) and [potential solution](#) in [troubleshooting](#)).

94. Determine the percentage of total fragments in the 200-1000 bp range and the average fragment size, using a smear analysis in the 200–1000 bp range.
 - a. Select one of the samples of interest and click on the tools icon in the top right corner of the Peak Table window.
 - b. Click on the tab Smear Analysis and select fragments between 200 bp and 1000 bp.
 - c. Apply the analysis settings to all samples of interest by selecting the appropriate option (i.e., apply to all or apply to selected) below the table.

Note: After applying the settings, results can be obtained by clicking the table icon in the top-right corner of the corresponding window. A new tab will appear with the Smear Analysis results. The average fragment size of the library is needed for quantification in step 105.

Library quantification and pooling

⌚ **Timing:** Day after purification of the final RNA sequencing library (i.e., day 3 or day 4). Note that this step can already be initiated while the individual sample libraries are being validated using the Fragment Analyzer system.

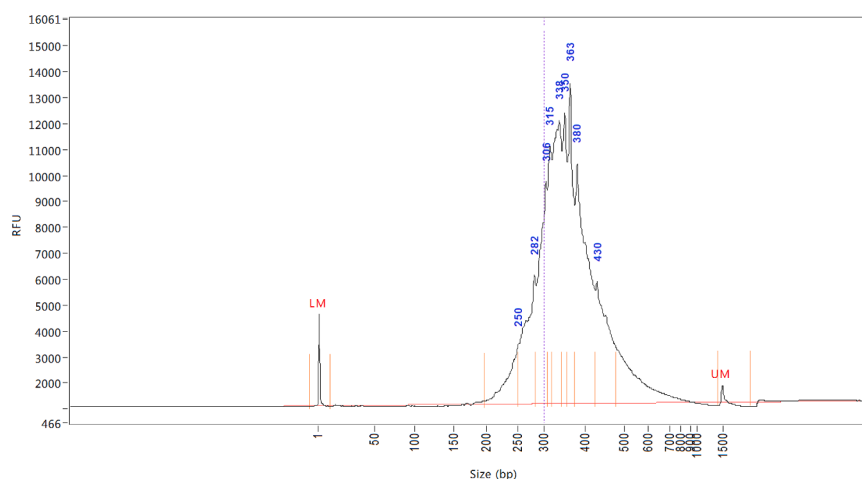


Figure 2. Expected Fragment Analyzer profile of a library prepared using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian

Fragment Analyzer profile of a good-quality library obtained from single-spun plasma from a patient diagnosed with diffuse large B-cell lymphoma (DLBCL).¹ A smear from 200 bp to 1000 bp with a peak around 300 bp is expected.

Individual libraries are quantified using the KAPA Library Quantification Kit and the LightCycler 480. Libraries are equimolarly pooled for sequencing. The experimental template in [File S2](#) can be used to set up the 384-well PCR plate layout for library quantification, to analyze these data and determine the individual library concentrations, to pool the individual libraries and quantify the library pool concentration, and to analyze these data and determine the individual library concentrations.

95. Thaw the master mix (as prepared in the Section [KAPA Library Quantification Kit](#)) and DNA Standards 1-6 at room temperature (18–22 °C).

Note: The KAPA SYBR FAST qPCR Master Mix is light sensitive. Protect from light during all protocol steps.

96. Thaw the library preparation plate at room temperature (18–22 °C).
97. Prepare four fresh 96-well PCR plates (dilution plate 1–4). Add LQD Buffer (prepared in the Section [sample dilution buffer for library quantification](#)) to the wells that correspond to wells that contain samples in the library preparation plate.
 - a. Dilution plate 1 (1/50 dilution): Add 98 μ L of LQD Buffer.
 - b. Dilution plate 2 (1/2000 dilution): Add 78 μ L of LQD Buffer.
 - c. Dilution plate 3 (1/80,000 dilution): Add 78 μ L of LQD Buffer.
 - d. Dilution plate 4 (1/400,000 dilution): Add 16 μ L of LQD Buffer.

Note: When using the VIAFLO 96, pour the LQD Buffer into a VIAFLO 96-compatible reagent reservoir, and add the appropriate buffer amount to dilution plate 1–4, with aspiration and dispense speeds set to 4. This can be done in variable-dispense mode (pre- and post-dispense volumes of 15 μ L) with a single set of pipetting tips.

98. Vortex and spin down the library preparation plate.
99. Add library to the dilution plates 1–4.
 - a. Dilution plate 1 (1/50 dilution):
 - i. Add 2 μ L of the library to obtain a total volume of 100 μ L.
 - ii. Seal the plate, vortex, and spin down.
 - b. Dilution plate 2 (1/2000 dilution):
 - i. Add 2 μ L from dilution plate 1 to obtain a total volume of 80 μ L.
 - ii. Seal the plate, vortex, and spin down.
 - c. Dilution plate 3 (1/80,000 dilution):
 - i. Add 2 μ L from dilution plate 2 to obtain a total volume of 80 μ L.
 - ii. Seal the plate, vortex, and spin down.
 - d. Dilution plate 4 (1/400,000 dilution):
 - i. Add 4 μ L from dilution plate 3 to obtain a total volume of 20 μ L.
 - ii. Seal the plate, vortex, and spin down.
100. Prepare two fresh 384-well PCR plates according to the sheet 'qPCR plate layout' in [File S2](#) to quantify the 1/80,000 (qPCR plate 1) and 1/400,000 (qPCR plate 2) library dilutions.
101. Add 3 μ L of master mix (step 95) to qPCR plate 1 and 2 to wells containing sample libraries, or DNA Standards 1-6 of the KAPA Library Quantification Kit or NTC. Seal the plates and spin down.
 - a. Multichannel pipetting option: When using an electronic multichannel pipette, use the multidispense function with an aliquot volume of 3 μ L, an aliquot number of 6, and aspiration and dispense speeds of 3.
 - b. Mantis option.
102. Add 2 μ L of library dilution plate 3 to qPCR plate 1, and 2 μ L of library dilution plate 4 to qPCR plate 2, as well as 2 μ L of the DNA Standards 1–6 and NTC to the corresponding wells, to obtain 5 μ L qPCR reactions.

Table 10. Protocol step 104: LightCycler 480 program for library quantification

Steps	Analysis mode	Cycles	Temperature	Acquisition mode	Time	Ramp rate (°C/s)	Acquisitions (per °C)
Enzyme Activation	None	1	95°C	None	5 min	4.8	None
Amplification—	Quantification—	35—	95°C	None	30 s	4.8	None
			60°C	Single	45 s	2.5	None
Melt curve—	Melting curves—	1—	95°C	None	5 s	4.8	None
			60°C	None	1 min	2.5	None
			95°C	Continuous	None	0.11	5
Cooling	None	1	40°C	None	3 min	2.5	None

Estimated time: ± 75 min.

- a. Multichannel pipetting option: When using an electronic multichannel pipette, use the multidispense function with an aliquot volume of 2 µL, an aliquot number of 3, and aspiration and dispense speeds set to 3.
 - b. VIAFLO 96 option: Add the libraries to the qPCR plates using the repeat dispense function, with a pre- and post-dispense volume of 2 µL and aspiration and dispense speeds set to 4.
103. Seal the plates, vortex, and spin down.

Note: Store the qPCR plates at –20°C if it is not possible to immediately proceed to step 104.

104. Run the qPCR plates on a LightCycler480 platform according to the program in [Table 10](#) and detection format SYBR Green I/HRM Dye.
105. Use the C_q values and average fragment sizes of the libraries (determined using the Fragment Analyzer software in Section [library validation](#), step 94) to calculate the average individual library concentrations using [File S2](#).
106. To equimolarly pool the individual libraries, dilute the libraries to the lowest concentrated individual library.

Note: Be sure not to dilute the libraries below the minimum concentration and volume needed for loading into the Illumina sequencing run ([Table 11](#)). Samples with library concentrations below this minimum loading concentration and volume need to be either excluded from pooling or added to the maximum volume. Note that the latter will likely yield fewer sequencing reads for these samples.

- a. Fill in the library volume to be used for the dilution (usually 2 µL is sufficient) and evaluate the required Pooling Buffer volume for each sample (in the sheet ‘Pooling’ in [File S2](#)).

Note: Make sure that the total volume of the individual diluted libraries does not exceed 200 µL (see Section [problem 3](#) and [potential solution](#) in [troubleshooting](#)).

- b. Add the appropriate volumes of Pooling Buffer to a fresh 96-well PCR plate.

Note: The Mantis Liquid Dispenser can be used for this step. Do not mix up the LQD Buffer (used in step 97) and the Pooling Buffer (used in step 106). The Pooling Buffer does not contain Tween20.

107. Add the appropriate volume of sample libraries to the 96-well PCR plate.
- a. Multichannel pipetting option.
 - b. VIAFLO 96 option: Add the libraries to the qPCR plates using a pre- and post-dispense volume of 2 µL.
108. Seal the plate, vortex, and spin down.
109. Pipette an identical volume from each library into a 1.5 mL LoBind tube.

Note: This volume depends on the concentration of the pool, the number of individual libraries, and the Illumina equipment selected for sequencing. Usually, it ranges between 3

Table 11. Illumina sequencers and flow cells, and corresponding loading concentrations, volumes, and %PhiX

Sequencer - flow cell ^a	Read lengths	Minimum concentration (pM)	Minimum volume (μL)	%PhiX
NextSeq 2000 - P2	2 × 50 bp, 2 × 100 bp	650	20	2
NovaSeq 6000 - SP	2 × 50 bp, 2 × 100 bp	580	100	2
NovaSeq 6000 - S1	2 × 100 bp	580	100	2
NovaSeq 6000 - S2	2 × 100 bp	580	150	2

^aThis overview is limited to sequencers, flow cells, and read lengths used so far by the OncoRNALab to perform total RNA sequencing on liquid biopsies, using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian or the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit for library preparation.

and 5 μL (in the sheet 'Pooling' in [File S2](#)). Ensure the final pool volume is sufficient to load onto the Illumina equipment selected for sequencing ([Table 11](#)).

- a. Multichannel pipetting option:
 - i. Transfer an identical volume from every row of the 96-well PCR plate into a well strip.
 - ii. Pool the wells from the strip into a 1.5 mL LoBind tube.

Note: Make sure to transfer all the content from the wells.

110. Validate the library pool using the Fragment Analyzer System, as described in the Section [library validation](#).

Note: The average fragment size of the library pool is needed for quantification in step 111 (see Section [problem 2](#) and [potential solution](#) in [troubleshooting](#)).

111. Perform three concentration measurements of the final library pool using a Qubit 4 Fluorometer and 1X dsDNA HS Assay Kit to calculate the mean library pool concentration (in the sheet 'Final Pool' in [File S2](#)).

Library size selection (optional)

⌚ **Timing:** Day after library quantification and pooling (i.e., day 4 or day 5)

Library size selection using the Pippin Prep System and Pippin Gel Cassette should only be performed in the presence of adapter dimers (see Section [problem 2](#) and [potential solution](#) in [troubleshooting](#)) to increase the purity of the final library. This size selection step is performed according to the [manufacturer's operations manual](#) and is followed by an ethanol purification step.

112. Equilibrate the marker/loading solution mix and electrophoresis buffer at room temperature (18–22 °C).
113. Rinse the electrode array by filling the rinse cassette with deionized water, loading it onto the sample tray, closing the Pippin Prep lid, and incubating for 20 s.
114. Go to the editor tab of the Pippin Prep software to write a new protocol.
 - a. Select the correct cassette type and programming mode range with size ranging from 200 to 600 bp.
 - b. Use the setting 'off' in the reference lane indicator field.
 - c. Indicate the use of internal standards and save the protocol.
115. Calibrate the Pippin Prep optical system.
 - a. Place the calibration fixture into the optical nest and follow the instructions in the manufacturer's operations manual.
 - b. Remove the calibration fixture when the procedure is completed.
116. Prepare the cassette and perform a continuity test as described in the manufacturer's operation manual.

Note: The marker/loading solution mix is light sensitive. Protect from light.

117. Prepare a library pool aliquot of 150 μ L from the library pool obtained in Section [library quantification and pooling](#), step 109.
118. Vortex and spin down the marker/loading solution mix.
119. Add 50 μ L of marker/loading solution mix to the library pool aliquot. Vortex thoroughly and spin down.
120. Load the pool across the five different lanes and run the protocol as described in the manufacturer's operations manual.
121. Recover 200 μ L (pooled from the five different lanes) of size-selected library pool in a 1.5 mL LoBind tube as described in the manufacturer's operations manual.
122. Pre-chill a microcentrifuge at 4 °C.
123. Pre-chill 5 mL 100% ethanol at –20 °C.
124. Add 2 μ L of glycogen, 30 μ L of 3 M sodium acetate, and 977 μ L of chilled ethanol to the size-selected library pool, and immediately centrifuge at 20,000 g for 20 min at 4 °C in the chilled microcentrifuge.
125. Pre-heat a thermomixer at 37 °C.
126. Prepare 2,500 μ L of 70% ethanol at room temperature (18–22 °C).
127. Remove and discard the supernatant, leaving the pellet intact.
128. Wash the pellet with 500 μ L of 70% ethanol at room temperature (18–22 °C).
129. Centrifuge at 20,000 g for 2 min at room temperature (18–22 °C).
130. Remove and discard the supernatant, leaving the pellet intact.
131. Dry the pellet by placing the tube with the lid open in the heated thermomixer for 5 to 10 min, or until dry.
132. Resuspend the pellet in 13 μ L of Pooling Buffer, as prepared in the Section [sample pooling buffer for library pooling](#).
133. Validate the library pool on the Fragment Analyzer as described in Section [library validation](#).

Note: Primer dimers should be removed, as demonstrated in [Figure 3](#) (see section [problem 2](#) and [potential solution](#) in [troubleshooting](#)).

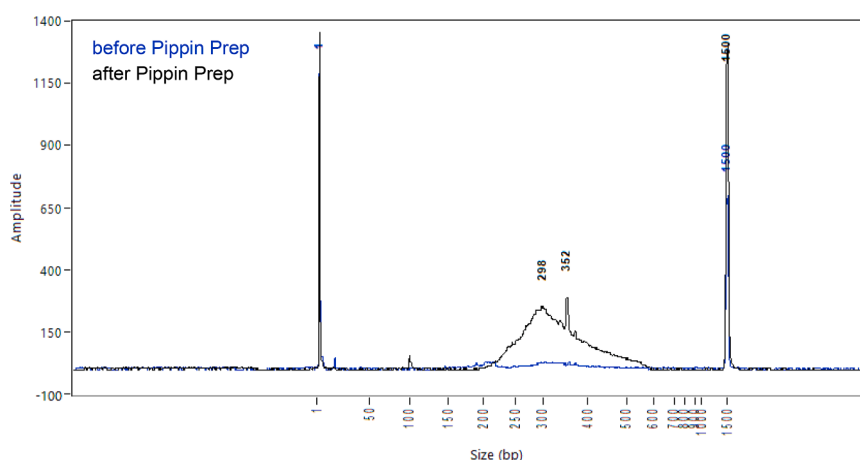


Figure 3. Fragment Analyzer profile of a library pool prepared using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian, demonstrating the presence of primer dimers

Fragment Analyzer profile of a library pool obtained from plasma of 80 IgM gammopathy patients. The peak at 125–200 bp before Pippin Prep demonstrates the presence of primer dimers. The Fragment Analyzer profile of the same library pool after Pippin Prep size selection and ethanol purification demonstrates the absence of primer dimers. Note that the peak at 100 bp is due to Pippin Prep reagents.

Sequencing

⌚ Timing: Hands-on time is 0.5–1 h, depending on the selected sequencing method.

134. Sequence the library pool on an Illumina instrument (NextSeq 500, HiSeq 2/3000, or NovaSeq 6000).

Note: For example, in Decruyenaere et al.,¹ the library was paired-end sequenced (2×100 nucleotides) on a NovaSeq 6000 instrument, using a NovaSeq S2 kit with standard workflow loading of 0.65 nM (2% PhiX).

EXPECTED OUTCOMES

Library validation

After Section [purification of the final RNA sequencing library](#), step 90, libraries such as the one presented in [Figure 2](#) are expected to be generated, with a smear from 200 bp to 1000 bp and a peak at approximately 300 bp.

Sequencing

An example of expected sequencing run output using equipment and run conditions indicated in [Table 11](#) is shown in [Table 12](#).

Table 12. Example of expected sequencing run output using equipment and run conditions indicated in Table 11

Sequencer - flow cell ^a	%PF	%Occupied	Reads PF (million)	%PhiX	%≥Q30
NextSeq 2000 - P2	77–83	82–94	516	4.62	91.31
NovaSeq 6000 - SP	68–82	96–98	939–1025	0.57–1.04	88.17–88.33
NovaSeq 6000 - S1	74–91	88–97	2090–2223	0.78–3.19	88.51–92.01
NovaSeq 6000 - S2	76–84	97–98	4618	1.19	89.99

^aThis overview is limited to sequencers and flow cells used so far by the OncoRNALab to perform total RNA sequencing on liquid biopsies, using the SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian or the SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) kit for library preparation. %PF: the percentage of clusters passing the filter that are assigned to the index. %Occupied: the percentage of wells (for patterned flow cells) or nonduplicated spots (for non-patterned flow cells) on the flow cell containing clusters. Reads PF: the number of clusters (in millions) passing filtering. %PhiX: the percentage of reads aligned to the PhiX genome. %≥Q30: the percentage of bases with a quality score of 30 or higher.

Data analysis

After data analysis, it is important to assess data quality, as outlined in the Section [quantification and statistical analysis](#) below. Four parameters are essential for this sequencing data: strandedness, splicing, spike-in fraction, and duplication rates (also see [problem 5](#) and [potential solution](#) in Section [troubleshooting](#)). To provide realistic values for these parameters, we selected the highest-quality samples from Decruyenaere et al.¹ ([Figure 4](#)). First, we expect stranded sequencing data from this library preparation. More specifically, we expect the vast majority of reads to map in the '1+-,1-+,2++,2--' orientation (following the RSeQC *infer_experiment* notation, [Figure 4A](#)). Second, although not all reads should cover splice junctions (since we are dealing with short-read sequencing), we expect about 10% of reads to map to known splice junctions ([Figure 4B](#)). Deviating values in these first two parameters might indicate DNA contamination of the RNA sample.²³ Third, the fraction of spike-in RNA in the final counts should be high enough to be useful for normalization without taking up too much of the data. Therefore, we recommend that the spike-in fraction (ERCC or Sequin) not exceed 5–10% ([Figure 4C](#)). Note that altered Sequin spike-in vs. ERCC spike-in ratios during data analysis, can indicate issues during RNA purification. Lastly, duplication rates indicate the degree of PCR duplicates and increase with a lower starting amount of RNA. Since the protocol works with low amounts of RNA, specifically, we expect a

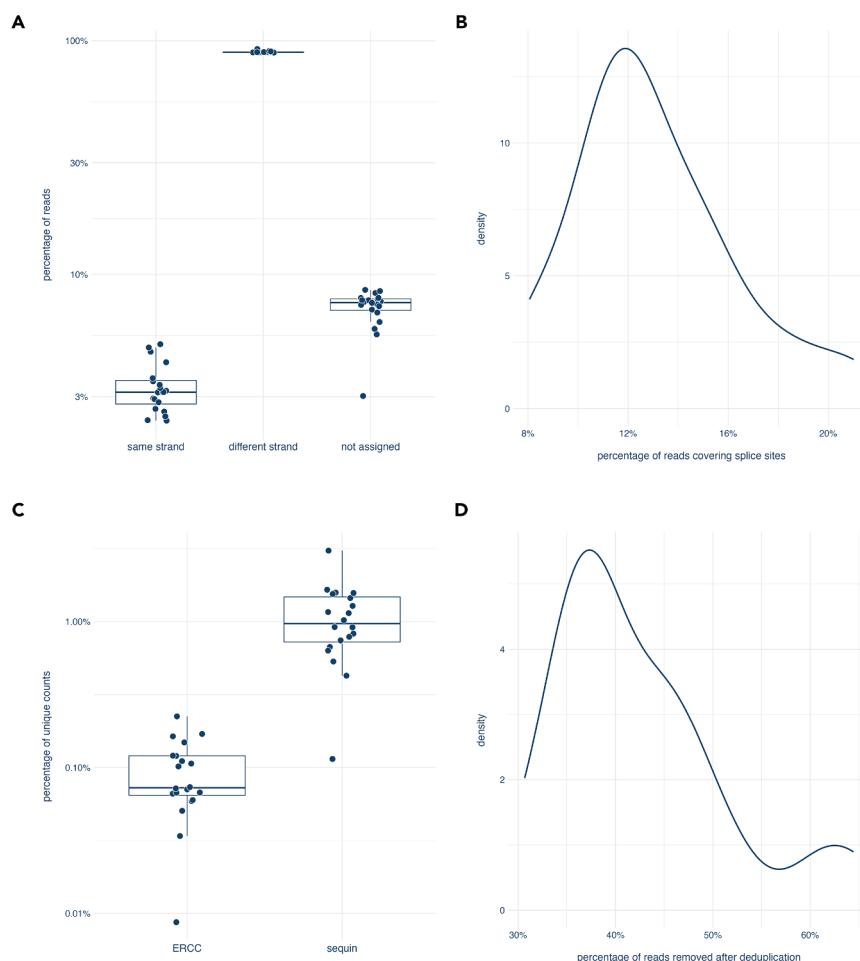


Figure 4. Quality control parameters for 20 high-quality samples from Decruyenaere et al.

(A) Representation of the output of *infer_experiment* from RSeQC.¹⁷ Each dot is a sample with outliers located outside the boxplot tails.

(B) Density plot showing the percentage of reads covering the splice sites. The x-axis is limited to the minimum and maximum values obtained in these data.

(C) Percentage of reads mapping to ERCC and Sequin spike-in RNA. Each dot is a sample with outliers located outside the boxplot tails. The y-axis is shown as a logarithmic scale.

(D) Density plot showing the percentage of reads removed after deduplication using *UMI-tools*.¹³ The x-axis is limited to the minimum and maximum values obtained in these data.

large proportion of PCR duplicates. Our high-quality samples show a mode of around 37% of duplicates. Extremely high duplication rates (>90%) indicate low RNA input (Figure 4D). Incorrect plasma preparation or poor blood tube choice may lead to an increased or decreased RNA concentration or integrity. This can also result in deviations in these last two parameters: unexpected spike-in RNA ratios and high duplication rates (Figures 4C and 4D).

QUANTIFICATION AND STATISTICAL ANALYSIS

When reviewing sequencing results, it is essential to carefully examine the quality control (QC) metrics before proceeding with downstream analyses. Illumina's BaseSpace platform (basespace.illumina.com) provides valuable run metrics, including the percentage of clusters passing filter that are assigned to the index (%PF), the percentage of wells (for patterned flow cells) or nonduplicated spots (for nonpatterned flow cells) on the flow cell containing clusters (%Occupied), the number of clusters (in millions) passing filtering (Reads PF), the percentage of reads aligned to

the PhiX genome (%PhiX) and the percentage of bases with a quality score of 30 or higher ($\geq Q30$). The %PF should be ideally above 75-80% and the %Occupied above 85% (see [Table 12](#) in Section [expected outcomes](#); see Section [problem 4](#) and [potential solution](#) in [troubleshooting](#)). For total RNA sequencing, we target approximately 20 million reads per sample for gene-level quantification or gene set enrichment analysis, and around 40 million reads per sample for more detailed investigations of low-abundance genes. Evaluate whether the proportion of reads aligning to the PhiX control corresponds to the concentration loaded during sequencing. Ideally, the $\geq Q30$ should be around 90% or higher. For a more detailed evaluation of sequencing quality, additional QC analysis should be performed using FastQC (v0.12.1).

Raw sequencing data can be processed using various tools. We use a comprehensive Snakemake workflow, available at Zenodo (Zenodo: <https://doi.org/10.5281/zenodo.19113165>). The workflow leverages multiple bioinformatics tools, each executed within its own conda environment to ensure reproducibility and ease of dependency management. It is adaptable to both single-end (SE) and paired-end (PE) sequencing data, and it supports both versions of the total RNA library preparation protocol. The data folders and parameters can be adjusted in the configuration file (config/config.yml), where the sequencing parameter specifies the sequencing type (either 'se' for single-end or 'pe' for paired-end).

Our proposed workflow for alignment-based analysis includes the following steps.

1. FASTQ files from different lanes are combined per sample and read end (R1 or R2 for paired-end sequencing data).
2. For paired-end sequencing data, UMIs are extracted from Read 2 using UMI-tools¹³ (v1.1.6) to facilitate UMI deduplication in step 4 below.
3. Reads are mapped to a reference genome using STAR¹⁴ (v2.7.11b), a splice-aware aligner. Resulting BAM files are indexed using SAMtools¹⁵ (v1.21).
4. Deduplication is performed according to the protocol and sequencing data type. For paired-end sequencing, deduplication is done based on UMIs with UMI-tools¹³ (v1.1.6). For single-end sequencing data where UMIs are not available in Read 1, Picard²⁴ (v3.3.0) can be used for duplicate removal.
5. Gene counts are determined with HTSeq¹⁶ (v2.0.5) in strand-specific mode, considering only uniquely mapped reads.
6. DNA contamination is evaluated based on strandedness and splicing information obtained with RSeQC¹⁷ (v5.0.4) as described by Verwilt et al.²³ An Rmarkdown (v.2.29) file is generated to provide an interactive view of these two metrics.
7. Overall quality is assessed using FastQC¹⁸ (v0.12.1), considering both raw sequencing data and aligned BAM files.

We recommend applying a threshold to filter out low-abundant, noisy genes. We typically set the cut-off at the count level that removes at least 95% of single-positive replicate values, following the method described by Mestdagh et al.²⁵

To demonstrate the pipeline's functionality and enable users to verify its compatibility with their infrastructure, test data (for both protocols and sequencing modes) is available in the 'tests' subfolder of the Zenodo repository. This includes (size-limited) sequencing data as well as intermediate and final output files.

LIMITATIONS

High-throughput sequencing platforms, including the Illumina system used here, require PCR amplification during library preparation to generate sufficient cDNA molecules for sequencing. In workflows optimized for biofluids, which are considered low-input samples, the number of PCR cycles is typically increased. This amplification step inevitably produces PCR duplicates – reads derived

from the same original cDNA molecule – which can bias downstream analyses if not adequately addressed. In the SMART-Seq Total RNA Pico Input with UMIs protocol, a Unique Molecular Identifier is incorporated during reverse transcription to mitigate PCR amplification bias. This UMI is positioned at the 5' end of Read 2, facilitating the identification of PCR duplicates in paired-end sequencing data. However, for single-end sequencing, this UMI is absent, rendering UMI-based deduplication unfeasible. Consequently, when processing single-end RNA sequencing data from biofluid samples, it is crucial to employ alternative computational tools to address PCR duplicates. Tools such as Picard (<http://broadinstitute.github.io/picard/>) or BMap's Clumpify utility (<https://sourceforge.net/projects/bbmap/>) can be utilized during the pre-processing stage to remove duplicates effectively. The expected percentage of duplicated reads varies depending on the tool used and the type of biofluid.

While our proposed computational workflow focuses on linear RNAs, circular RNAs can also be detected using this protocol. Quantification of circular RNAs is typically based on their characteristic backsplice junctions, and several specialized bioinformatics tools are available for this purpose.²⁶

TROUBLESHOOTING

Problem 1

During the purification steps, the wells of the library preparation plate contain AMPure XP beads, and the supernatant needs to be discarded (e.g., Section [purification of the RNA sequencing library](#), step 29) or transferred to a fresh 96-well PCR plate (e.g., Section [purification of the RNA sequencing library](#), step 39). Aspiration of the supernatant might result in the aspiration of (a small amount of) beads.

Potential solution

Two potential solutions are proposed, depending on whether the supernatant is discarded or transferred. (1) If beads are observed in the pipette tips during supernatant removal, the fluid should be pipetted back into the original 96-well PCR plate. Incubate the plate for 1 or 2 more min on the magnetic stand. Lower the volume to be removed by 3–5 μ L, and try again. Repeat if needed. Make sure that no beads are pipetted out. (2) If beads are observed in the pipette tips during supernatant transfer, the fluid also needs to be pipetted back into the original 96-well PCR plate. Then, add an additional volume (3–5 μ L) of PCR-grade water, ZMM or Tris Buffer of the library preparation kit to the wells, depending on the solution in which the beads were rehydrated. Incubate the plate for 1 or 2 more min on the magnetic stand. Try again to transfer the indicated volume and repeat if needed. Make sure that no beads are pipetted out. As such, transfer volumes and continuation of the protocol are secured.

Problem 2

During the 'Library validation' (Section [library validation](#), step 93) a small peak might appear before the expected library size. These peaks in the 125–200 bp range pinpoint the presence of primer dimers.

Potential solution

In that case, after pooling the individual libraries (Section [library quantification and pooling](#), step 109), the final library pool should be re-evaluated for primer dimers (example in [Figure 3](#)). To assess the amount of dimers present in the library pool, a smear analysis can be performed using the ProSize Fragment Analyzer data analysis software (Agilent) to determine the percentage of fragments in the 125–200 bp range (similarly as described in Section [library validation](#), step 94). When this percentage exceeds 15%, it is recommended to perform library size selection using Pippin Prep (Section [library quantification and pooling](#)).

Problem 3

Some individual library concentrations may be too high to be used immediately for pooling without exceeding the maximum well volume of a 96-well PCR plate (i.e., 200 μ L, Section [library quantification and pooling](#), step 106).

Potential solution

For these libraries, make a subdilution series from 4 to 10 nM. Then, further dilute to the selected pool concentration.

Problem 4

The %Occupied and %PF metrics deviate from the ideally obtained values (Section [quantification and statistical analysis](#)). This might indicate suboptimal loading concentrations during the sequencing run.

Potential solution

To optimize loading concentrations on the NextSeq 2000 or NovaSeq 6000 sequencing instrument, the %Occupied and %PF metrics should be evaluated in a Sequencing Analysis View (SAV) plot to determine if a run was underloaded, optimally loaded, or overloaded. If the resulting plot indicates the run is underloaded or overloaded, increase or decrease the loading concentration, respectively, to optimally load the library pool.

Problem 5

Quality control plots may deviate from the expected profiles described above ([Figures 4A–4D](#); see Section [data analysis in expected outcomes](#)). Such deviations do not necessarily invalidate the experiment but may indicate technical issues that require careful interpretation, exclusion of specific samples, or protocol optimization before continuing with downstream analyses.

Common issues observed include:

- Evidence of DNA contamination: Suggested by a reduced proportion of reads mapping to the expected stranded orientation ([Figure 4A](#)) and/or a substantially lower fraction of reads mapping to known splice junctions (typically <10%) ([Figure 4B](#)).
- Low spike-in fractions ([Figure 4C](#)): Spike-in RNA (ERCC or Sequin) is intended to support normalization and additional quality checks. If spike-in fractions are substantially below the recommended 5–10%, spike-ins may be insufficient for these purposes.
- Very high duplication rates ([Figure 4D](#)): These are expected when working with low RNA input, such as cell-free RNA from plasma. However, extremely high duplication rates (>90%) may indicate insufficient RNA input or over-amplification during library preparation.

Potential solution

Evidence of DNA contamination:

- Exclude clear outlier samples showing markedly reduced reads mapping to the expected strand and/or splice junctions, as these samples may not predominantly reflect RNA-derived signal.
- If many samples show similar deviations, this likely indicates an issue during the DNA removal step. In this case, repeat DNase treatment after verifying that the RNA purification kit used is compatible with the chosen DNA removal method.⁷

Low spike-in fractions:

- If spike-in counts are low but detectable, proceed with standard library-size normalization for downstream analyses.

- If spike-in reads are absent or extremely low, check spike-in dilution accuracy and confirm correct addition of spike-in RNA during library preparation.
- For subsequent experiments, consider using a less diluted spike-in mix to ensure adequate representation without excessively consuming sequencing depth.

Very high duplication rates.

- If duplication rates are high but comparable across samples, downstream analyses can still be performed, as biological comparisons remain valid under consistent technical conditions.
- If only a subset of samples shows extremely high duplication, consider excluding these samples due to reduced library complexity.
- For future experiments, consider increasing the RNA input where possible, reducing the number of PCR cycles, or optimizing library amplification conditions to improve library complexity.

RESOURCE AVAILABILITY

Lead contact

Requests for further information should be directed to the lead contact, Annelien Morlion (annelien.morlion@ugent.be).

Technical contact

Technical questions regarding the execution of this protocol should be directed to the technical contact, Annelien Morlion (annelien.morlion@ugent.be).

Materials availability

No newly generated materials are associated with this protocol.

Data and code availability

The accession number for the raw sequencing data (FASTQ files) reported in this paper is EGA: EGAS00001007585 (<https://ega-archive.org/studies/EGAS00001007585>).¹ These data are available under restricted access to comply with data privacy regulations. Access can be requested through EGA, and requests will be evaluated by the ethics committee of Ghent University Hospital (EGA: <https://ega-archive.org/datasets/EGAD00001011679>). Processed and pseudonymized count files are openly available through Zenodo (Zenodo: <https://doi.org/10.5281/zenodo.19113165>). All code and instructions for processing the data, as well as code for reproducing the manuscript figures, are also available on Zenodo.

ACKNOWLEDGMENTS

A.D. was supported by a senior postdoctoral fellowship grant from the Research Foundation Flanders (FWO; 1266325N) and by a grant from the Fund Suzanne Duschene, managed by the King Baudouin Foundation Belgium. A.M. was supported by a predoctoral fellowship grant from the FWO (11C1623N) and a postdoctoral fellowship grant from the Belgian Foundation Against Cancer (2025-085). J.V. was supported by an "opvangmandaat FWO postdoc" fellowship grant from BOF Antwerp University (52065) and a junior postdoctoral fellowship grant from the FWO (12A6F26N). P.D. was funded by the FWO (11H7523N). This work was also supported by GOA BOF Ghent University, the Belgian Foundation Against Cancer, and Kom Op Tegen Kanker (Stand Up To Cancer, the Flemish Cancer Society). The computational resources (Stevin Supercomputer Infrastructure) and services used in this work were provided by the VSC (Flemish Supercomputer Center), funded by Ghent University, FWO, and the Flemish Government – department EWI. The graphical abstract includes public-domain illustrations from NIH BioArt Source (NIAID Visual & Medical Arts, 10/7/2024: BDBlood Vial, bioart.nih.gov/bioart/52 & Next Gen Sequencer, bioart.nih.gov/bioart/386). The authors acknowledge financial support from Takara Bio for covering the publication fees of this article. The sponsor had no role in the selection or description of the methodologies presented, and the decision to submit the manuscript for publication was made independently by the authors.

AUTHOR CONTRIBUTIONS

Conceptualization, A.D.; data curation, A.M. and J.V.; formal analysis, A.M. and J.V.; funding acquisition, A.D.; investigation, K.V.; methodology, A.D. and K.V.; project administration, A.D.; software, A.M. and J.V.; resources, A.D., A.M., J.V., K.V., and P.D.; supervision, A.D., A.M., and J.V.; validation, K.V.; visualization, A.D., A.M., J.V., and K.V.; writing – original draft, A.D., A.M., J.V., and K.V.; writing – review and editing, A.D., A.M., J.V., K.V., and P.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2026.104540>.

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