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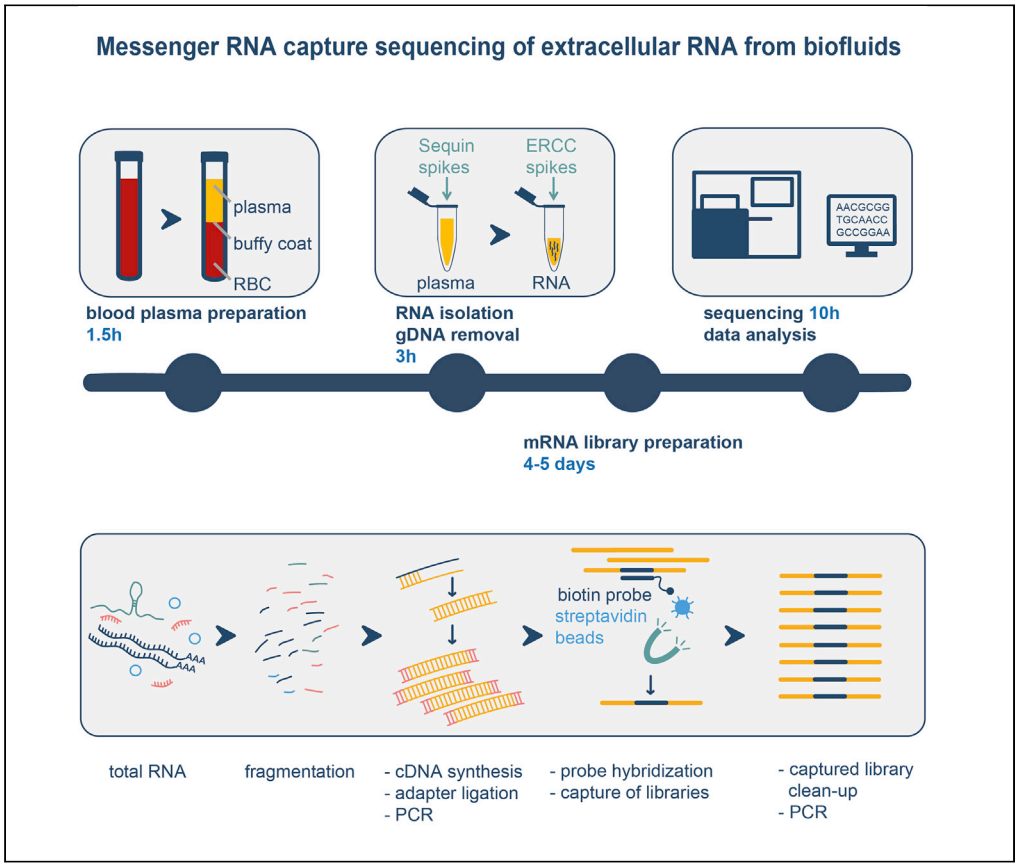
Messenger RNA capture sequencing of extracellular RNA from human biofluids using a comprehensive set of spike-in controls



Comprehensive transcriptome analysis of extracellular RNA (exRNA) purified from human biofluids is challenging because of the low RNA concentration and compromised RNA integrity. Here, we describe an optimized workflow to (1) isolate exRNA from different types of biofluids and (2) to prepare messenger RNA (mRNA)-enriched sequencing libraries using complementary hybridization probes. Importantly, the workflow includes 2 sets of synthetic spike-in RNA molecules as processing controls for RNA purification and sequencing library preparation and as an alternative data normalization strategy.

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Highlights

Extracellular RNA from biofluids has a low concentration and a compromised integrity

An optimized workflow for mRNA capture sequencing of human biofluids is provided

Synthetic spike-in RNA molecules serve as processing controls

Spike-in RNAs allow for data normalization and calculation of mRNA concentration

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Messenger RNA capture sequencing of extracellular RNA from human biofluids using a comprehensive set of spike-in controls

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SUMMARY

Comprehensive transcriptome analysis of extracellular RNA (exRNA) purified from human biofluids is challenging because of the low RNA concentration and compromised RNA integrity. Here, we describe an optimized workflow to (1) isolate exRNA from different types of biofluids and (2) to prepare messenger RNA (mRNA)-enriched sequencing libraries using complementary hybridization probes. Importantly, the workflow includes 2 sets of synthetic spike-in RNA molecules as processing controls for RNA purification and sequencing library preparation and as an alternative data normalization strategy.

For complete details on the use and execution of this protocol, please refer to Hulstaert et al. (2020).

BEFORE YOU BEGIN

The protocol below describes the blood collection method and blood plasma preparation protocol to collect platelet-rich (PRP), platelet-poor (PPP) and platelet-free plasma (PFP) as defined in Hulstaert et al (Hulstaert et al., 2020). However, we also successfully applied the entire workflow in the step-by-step method details section to the extracellular supernatant of a wide range of human biofluids, i.e., amniotic fluid, aqueous humor, ascites, bile, bronchial lavage fluid, breast milk, cerebrospinal fluid, colostrum, gastric fluid, pancreatic cyst fluid, saliva, seminal plasma, serum, sputum, stool, synovial fluid, sweat, tear fluid and urine.

Details on the biofluid collection and sample preparation of these liquid biopsies are described in Hulstaert et al. (2020).

Blood collection

Timing: ~15 min

1. Human venous blood is collected from an elbow vein after disinfection with 2% chlorhexidine in 70% alcohol. The blood draw is performed with a butterfly needle of 21 gauge (Cat# 367326, Becton Dickinson and Company) and 10 mL K2-EDTA tube (Cat# 367525, Becton Dickinson and Company). Blood collection tubes are filled to the volume recommended by the manufacturer.
2. Immediately after the blood draw, blood collection tubes are inverted five times to mix the anti-coagulant with the blood and transported upright in an isolated transportation box at room temperature (18°C–22°C) to the laboratory for further processing.

Note: Pre-analytical variables, such as the type of blood collection tube, collection method, time between biofluid collection and processing, and storage of the extracellular supernatant at −80°C, impact the results obtained in further analyses (Decock et al., 2020). Therefore, we strongly recommend making use of uniformly collected series of biofluid samples, and to keep track of all details regarding pre-analytical variables (Grossman et al., 2017).

Platelet-rich, -poor and -free plasma preparation

Timing: ~1.5h

A schematic overview of the plasma preparation protocol is provided in Figure 1.

3. All centrifugation steps during the plasma preparation protocol are performed at room temperature (18°C – 22°C) in a centrifuge with swinging bucket rotor and appropriate adapters. No or minimal acceleration and no braking should be applied. Before initiating the first centrifugation step, all blood collection tubes (Cat# 188271, Greiner Bio-One International) are inverted five times.
4. Platelet-rich plasma (PRP) is obtained after centrifugation of blood tubes at 400 × *g* for 20 min. Carefully transfer the plasma using a 1 mL pipet to a 15 mL conical tube leaving approximately 0.5 cm above the buffy coat.

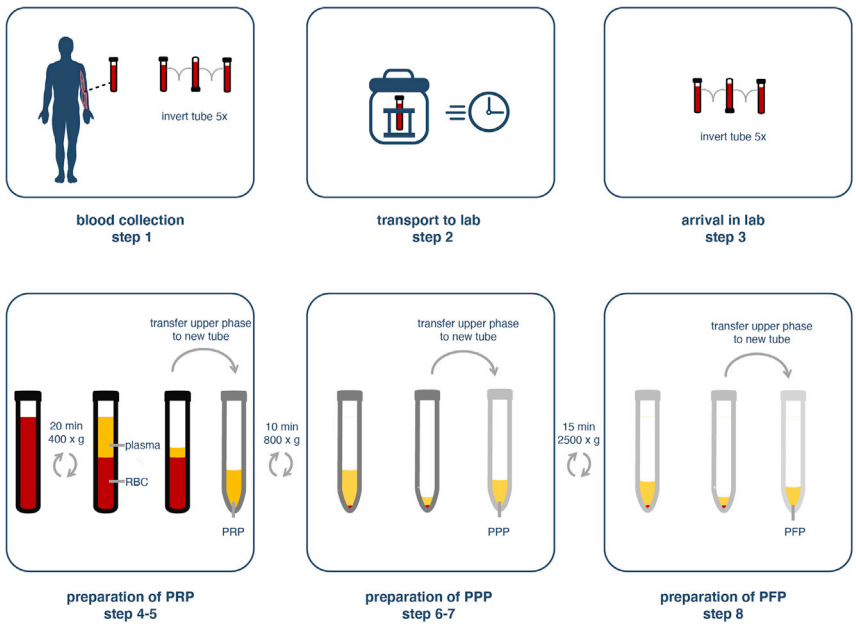

Figure 1. Three-spin plasma preparation protocol
PRP: platelet-rich plasma; PPP: platelet-poor plasma; PFP platelet-free plasma; RBC: red blood cells.

5. If PRP is needed for further experiments, aliquot PRP per 200 μL into LoBind 1.5 mL tubes (Cat# Z666556-250EA, Eppendorf AG), snap freeze in liquid nitrogen and subsequently store at −80°C.
6. Remaining PRP can be used for a second spin at 800 × *g* for 10 min. Platelet-poor plasma (PPP) is then carefully transferred to a fresh 15 mL tube leaving approximately 0.5 cm above the platelet pellet.
7. PPP can be aliquoted per 200 μL into LoBind tubes, snap frozen in liquid nitrogen and subsequently stored at −80°C or further processed.
8. To obtain platelet-free plasma (PFP), PPP is spun at 2500 × *g* for 15 min. PFP is carefully transferred to a fresh 15 mL tube leaving approximately 0.5 cm above the platelet pellet. Aliquot PFP per 200 μL into LoBind tubes, snap freeze in liquid nitrogen and store at −80°C.

⚠ CRITICAL: To avoid post-collection changes in the exRNA profile, we advise to process all blood collection tubes within 2 h after collection.

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.

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

Note: Using the aforementioned protocol, a completely filled 10 mL blood tube will result in a yield of approximately 3–5 mL PRP, depending on the donor.

Note: If the ultimate goal is the preparation and usage of platelet-free plasma, we recommend a 2-step protocol, with at least one centrifugation step of 15 min at 2500 × *g*.

Note: Assess the degree of hemolysis, a known pre-analytical factor that might impact the final sequencing results.(Kirschner et al., 2011, 2013) The rupture of red blood cells releases hemoglobin in the plasma, changing the plasma from being relatively colorless to having a pale pink to red hue. The degree of hemolysis can be measured by visual inspection after centrifugation or, more accurately, by measuring the specific light absorbance of hemoglobin at 414 nm using a spectrophotometer (e.g., NanoDrop 1000, Thermo Fisher Scientific).(Shah et al., 2016; Van Buren et al., 2020)

Dilution of RNA spike-in controls

Timing: ~1h

9. Prepare Sequin spike-in controls aliquots by diluting the Sequin stock (Garvan Institute of Medical Research, www.sequinstandards.com) in RNase-free water (Cat# W4502, Sigma) as indicated in Table 1.
10. Prepare External RNA Control Consortium (ERCC) spike-in controls aliquots by diluting the ERCC stock (Cat# 4456740, Thermo Fisher Scientific) in RNase-free water as indicated in Table 2.

An overview of the Sequin and ERCC spike-in controls with their stock concentration and length is provided in Table S1: Sequin and ERCC spike-in controls, related to step 10.

Note: ERCC and Sequin stocks should be stored in single-use aliquots at −80°C. We recommend storage for up to 1 year. Longer storage is likely possible but should be verified by the user.

Table 1. Dilution steps to prepare Sequin spike-in controls working solution

Solution name	Solution volume	RNase-free water	Final dilution (relative to stock)
Sequin stock (15 ng/μL)	NA	NA	1
Sequin working solution a	2 μL of Sequin stock	198 μL	1:100
Sequin working solution b	2 μL of Sequin working solution a	198 μL	1:10 000
Sequin working solution c	7.7 μL of Sequin working solution b	993.3 μL	1:1 300 000

Preparation of buffer working solutions for RNA purification

Timing: ~10 min

11. Buffers RWT and RPE are supplied as concentrates in the miRNeasy Serum/Plasma Kit (Cat# 217184, Qiagen). Before using for the first time, follow the steps in Table 3 to obtain a working solution. We use absolute ethanol (Cat# 20821.321, VWR Chemicals) to prepare the buffer working solutions and to prepare 80% ethanol throughout the workflow.

Preparation of anti-CEX oligo pool

Timing: ~10 min

12. Prepare 100 μM stock solutions of anti-CEX oligonucleotides (ordered from Integrated DNA Technologies, Table S2: Oligos to capture spike-in controls, related to step 12). Pool 5 μL of a 100 μM solution of each oligo (34 in total, listed in Table 6) to obtain a 2.94 μM anti-CEX oligo pool. Use 20 μL of the 2.94 μM anti-CEX oligo pool and add 450.6 μL of a 10 mM Tris-HCl buffer (pH 8.5) to create a 1000× anti-CEX oligo pool, as shown in Table 4.

Note: The anti-CEX oligo pool should be stored at −20°C. We recommend storage for up to 1 year. Longer storage is likely possible but should be verified by the user.

Preparation of capture probe pool for spike-in controls

Timing: ~10 min

13. Prepare a 70.4 ng/μL stock solution of biotinylated 80-mer capture probes complementary to the spike-in controls (ordered from Twist Biosciences, Table S2: Oligos to capture spike-in controls, related to step 12) by adding 482 μL of 10 mM Tris-HCl buffer (pH 8.5) to 34 μg lyophilized probes (corresponding to an order of 96 reactions), resulting in a concentration of 4 nM per probe, as shown in Table 5. The anti-CEX oligo pool ensures that globin, which may be abundant in biofluids, is not captured during the library preparation protocol.

Note: The probe pool should be stored at −80°C. We recommend storage for up to 1 year. Longer storage is likely possible but should be verified by the user.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Biofluid sample	Human donor	N/A
Critical commercial assays		
Absolute ethanol	VWR Chemicals	Cat# 20821.321

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
BD Vacutainer, Push Button Blood Collection Set	Becton Dickinson and Company	Cat# 367326
BD Vacutainer K2-EDTA tube	Becton Dickinson and Company	Cat# 367525
RNase AWAY Surface Decontaminant	Thermo Fisher Scientific	Cat# 7000TS1
RNase-free water	Sigma	Cat# W4502
miRNeasy Serum/Plasma Kit	QIAGEN	Cat# 217184
QIAzol Lysis Reagent	QIAGEN	Cat# 217184
RNeasy MinElute spin column	QIAGEN	Cat# 217184
QIAamp ccfDNA/RNA Kit	QIAGEN	Cat# 55184
Chloroform	Sigma-Aldrich	Cat# 372978-1L; CAS: 67-66-3
External RNA Control Consortium (ERCC) spike-in controls, mix 1	Thermo Fisher Scientific	Cat# 4456740
10× reaction buffer for gDNA removal	ArcticZymes AS	Cat# 66001
HL-dsDNAse for gDNA removal	ArcticZymes AS	Cat# 70800-202
TruSeq RNA Library Prep for Enrichment (including First Strand Synthesis Act D Mix, PCR Master Mix, PCR Primer Cocktail, Second Strand Marking Master Mix, Elution Primer Fragmentation Mix, Ligation Mix, A-Tailing Mix, Stop Ligation Buffer, Resuspension Buffer)	Illumina	Cat# 20020189
TruSeq RNA Enrichment (including Capture Target Buffer 3, Enrichment Elution Buffer 1, Enhanced PCR Mix, Enrichment Wash Solution, 2N NaOH, PCR Primer Cocktail, Resuspension Buffer)	Illumina	Cat# 20020490
Exome Panel – Enrichment Oligos (including Elute Buffer 2, Streptavidin Magnetic Beads)	Illumina	Cat# 20020183
TruSeq RNA Single Indexes Set A and B or TruSeq RNA CD Index Plate	Illumina	Cat# 20020492; Cat# 20020493; Cat# 20019792
SuperScript II Reverse Transcriptase 2000 U/µL	Thermo Fisher Scientific	Cat# 18064014
AMPure XP beads	Beckman Coulter	Cat# A63881
HS Small Fragment Kit	Agilent	Cat# DNF-477-0500
KAPA Library Quantification Kit	Roche Diagnostics	Cat# 07960140001
10 mM Tris-HCl buffer pH 8.5	Bioworld	Cat# 42020414-1
PhiX control V3 (	Illumina	Cat# FC-110-3001
NextSeq 500/550 HO Kit v2.5 150 Cycles	Illumina	Cat# 20024907
Deposited data		
Raw data	European Genome-Phenome Archive (EGA)	EGAS00001003917
Software and algorithms		
FastQC software (v0.11.5)	Babraham Institute	RRID:SCR_014583
STAR (v2.6.0) Ultrafast Universal RNA-seq Aligner	(Dobin et al., 2013)	RRID:SCR_015899
Picard (v2.18.5)	Broad Institute	RRID:SCR_006525
HTSeq (v0.9.1)	EMBL Heidelberg	RRID:SCR_005514
Custom pipeline for mRNA capture seq analysis	Hulstaert et al., 2020	https://github.com/OncoRNALab/RNAexome
Other		
Sequin spike-in controls, mix A	Garvan Institute of Medical Research, Hardwick et al., 2016	www.sequinstandards.com
Anti-CEX oligonucleotides	Integrated DNA Technologies	www.idtdna.com
Capture probe pool for spike-in controls	Twist Biosciences	www.twistbioscience.com/products/oligopools

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
15 mL Conical tube, CELLSTAR polypropylene tube	Greiner Bio-One International	Cat# 188271
2 mL LoBind microcentrifuge tubes	Eppendorf AG	Cat# Z666556-250EA
Non-skirted standard 96-well plate and adhesive seal	Thermo Fisher Scientific	Cat# SP-0074, Cat# SP-0027, Cat# SP-0028, Cat# SP-0605
Low-profile 8-strip PCR tubes	Thermo Fisher Scientific	Cat# SP-0085
Eppendorf Thermomixer Comfort	Eppendorf AG	Cat# 460-1112
T100 Thermal Cycler	Bio-Rad	Cat# 186-1096
Cooling unit	VWR International	Cat# 89004-558

MATERIALS AND EQUIPMENT

Sequin and External RNA Control Consortium (ERCC) spike-in controls

Sequin spike-in controls (Garvan Institute of Medical Research, mix A) are added to the biofluid lysate prior to further RNA purification (Hardwick et al., 2016). An overview of the concentration of each individual Sequin spike-in is provided in Table S1: Sequin and ERCC spike-in controls, related to step 10. A volume of 2 μL Sequin working solution c (Table 1) is added to the lysate of a 200 μL biofluid sample.

ERCC spike-in controls (Cat# 4456740, mix 1, Thermo Fisher Scientific) are added to the RNA eluate, before gDNA removal. A volume of 2 μL ERCC working solution c (Table 2) is added to 12 μL RNA eluate. An overview of the ERCC spikes and stock concentrations is provided in Table S1: Sequin and ERCC spike-in controls, related to step 10. The mentioned spike concentrations are optimized for our study. We recommend to end up with 2.5% of the reads mapping to each spike-in set. The spike solutions should be stored at −80°C. We recommend storage for up to 1 year. Longer storage is likely possible but should be verified by the user.

Anti-CEX oligonucleotides

During the two hybridization steps of the TruSeq RNA Exome library preparation 1.25 μL of an anti-CEX oligo pool is added. These oligos ensure that abundant globin mRNAs in biofluids are not captured in the final sequencing library (Table 6).

Capture probes for spike-in controls

To detect the spike-in controls, the capture probes of the TruSeq RNA Exome kit are complemented with 0.625 μL of the 70.4 ng/μL stock solution of the capture probes for both the Sequin and ERCC spike-in sets (2.5 fmol/probe/reaction) in the first and second hybridization step of the library preparation protocol. These probes are 80-mers designed by tiling the spike-in sequences and do not map to the human genome. To this purpose, only 80-mers with a GC content between 25%–70%, a GC-based Tm between 60°C–80°C and a ΔG larger than -7 (calculated by UNAFold version 3.8 settings: hybrid-ss-min -E -n DNA -t 54 -T 54) were retained, and further filtered to end up with 3560 probes, i.e., the minimal number of probes needed to obtain optimal spike-in coverage (Markham and Zuker, 2008). The sequences of these oligos are provided in Table S2: Oligos to capture spike-in controls, related to step 12.

Table 2. Dilution steps to prepare ERCC spike-in controls working solution

Solution name	Solution volume	RNase-free water volume	Final dilution (relative to stock)
ERCC stock	NA	NA	1
ERCC working solution a	2 μL of ERCC stock	198 μL	1:100
ERCC working solution b	2 μL of ERCC working solution a	198 μL	1:10 000
ERCC working solution c	10 μL of ERCC working solution b	990 μL	1:1 000 000

Table 3. Steps to prepare buffer working solutions for RNA purification	
RWT working solution	add 2 volumes of ethanol (96%–100%) to the provided RWT concentrate
RPE working solution	add 4 volumes of ethanol (96%–100%) to the provided RPE concentrate

STEP-BY-STEP METHOD DETAILS

RNA purification

Timing: ~2.5 h for 12 samples

This step describes the protocol for total RNA purification starting from 200 µL biofluid using the miRNeasy Serum/Plasma Kit (Cat# 217184, Qiagen).

1. Biofluid samples of 200 µL are thawed on a cooling unit (Cat# 89004-558, VWR International) and 1000 µL QIAzol Lysis Reagent (Cat# 217184, Qiagen) is added to each sample.
2. Samples are vortexed for 5 s and subsequently incubated for 5 min at room temperature (18°C – 22°C), followed by the addition of 2 µL Sequin working solution c.
3. Subsequently, samples are vortexed and 200 µL chloroform (Cat# 372978-1L, CAS: 67-66-3, Sigma-Aldrich) is added, followed by vortexing of the lysates for 15 s.
4. After a 2 min incubation at room temperature (18°C – 22°C), samples are centrifuged for 15 min at 12000 xg at 4°C. The lysate is now separated into 3 phases, as shown in Figure 2. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins end up in both the lower organic phase and the interphase.
5. Next, 600 µL of the upper aqueous phase is transferred to a new collection tube on a cooling unit, to which 900 µL ethanol is pipetted. Carefully transfer the upper, aqueous phase with a 200 µL pipet and avoid disturbance of the white interphase. **Troubleshooting 1**
6. Samples are thoroughly mixed by pipetting up and down, loaded (up to 700 µL) on a RNeasy MinElute spin column and centrifuged for 15 s at 10000 × *g* at room temperature (18°C – 22°C). The flowthrough is discarded and loading and centrifugation repeated using the remainder of the samples.
7. Afterwards, 700 µL RWT buffer is added to the column and samples are centrifuged for 15 s at 10000 × *g*. Flowthroughs are discarded.
8. 500 µL RPE buffer is pipetted onto the columns, followed by centrifugation for 15 s at 10000 × *g*. Again, flowthroughs are discarded.
9. 500 µL 80% ethanol is loaded onto the columns. Samples are centrifuged for 2 min at 10000 × *g*, and afterwards, the columns are placed into a new collection tube (with open lid) and dried for 5 min at full speed (16900 × *g*).
10. Finally, the columns are placed in a new collection tube, 14 µL RNase-free water is added to the center of the column membrane, and RNA is eluted by centrifugation for 1 min at full speed (16000 × *g* – 21000 × *g*). An RNA eluate of 12 µL should be obtained. **Troubleshooting 2**
11. Transfer 12 µL of the RNA eluate to a new collection tube on a cooling unit.

⚠ CRITICAL: To avoid degradation of RNA, create and maintain an RNase-free environment for the entire sample preparation and RNA purification procedure. Ribonucleases (RNases) are stable and active enzymes that are difficult to inactivate. Even small amounts of RNases are sufficient to degrade RNA. Do not use any plastic- or glassware without first eliminating possible RNase contamination. A proper aseptic technique should always be maintained

Table 4. Steps to prepare a 1000× anti-CEX oligo pool		
Reagent	final concentration	Amount
2.94 µM anti-CEX oligo pool	1000× anti-CEX oligo pool	20 µL
10 mM Tis-HCl pH 8.5	9.58 mM	450.6 µL

Table 5. Steps to prepare a capture probe pool for spike-in controls	
70.4 ng/μL stock solution of capture probe pool for spike-in controls	add 482 μL Tris-HCl pH 8.5 to 34 μg lyophilized probes

when working with RNA. Bacteria or molds from the surface of the skin or from dusty laboratory equipment can result in inadvertent RNase contamination. Always wear gloves when working with RNA samples and keep tubes closed whenever possible. Regularly decontaminate the bench surface, glassware, plasticware, and laboratory apparatus with RNase AWAY Surface decontaminant (Cat# 7000TS1, ThermoFisher Scientific). Note that when working with chemicals, such as chloroform, a suitable lab coat, disposable gloves, protective goggles, and working in a fume hood are necessary. For more detailed information, consult the appropriate material data sheets, available from the product supplier.

Pause point: We advise to continue directly with gDNA removal on the RNA eluate to avoid an unnecessary freeze/thaw cycle. However, the RNA eluate can be safely stored at −80°C and thawed on a cooling unit immediately before gDNA removal. Please ensure the number of freeze/thaw cycles is identical for all samples of the same project.

Table 6. Composition of the 1000× anti-CEX pool

anti-coding exome oligo	Sequence
HBA1_1_CEX_T	GGCCTTGACGTTGGTCTTGTCGGCAGGAGACAGCACCATGGTGGGTTCTCTCTGAGTCTGTGGGGACCAGAAGAGTGCCG
HBA1_2_CEX_T	GCCGACCTTACCCCAGGCGGCCTTGACGTTGGTCTTGTCGGCAGGAGACAGCACCATGGTGGGTTCTCTCTGAGTCTGTG
HBA1_3_CEX_T	GAGGGAGCCTCACCTCTCCAGGGCCTCCGCACCATACTCGCCAGCGTGCGCGCCGACCTTACCCCAGGCGGCCTTGACGT
HBA1_4_CEX_T	CGGGCGAGGAGCCCGGGTCGGAGCAGGGGAGGGAGCCTCACCTCTCCAGGGCCTCCGCACCATACTCGCCAGCGTGCGCG
HBA1_5_CEX_T	GGGAAGGACAGGAACATCCTGCGGGGAGAAGCAGAGTGAGGGGTGGGGTTTGGGTCCGGGGCCAGGACGGTTGAGGGTGG
HBA1_6_CEX_T	GGGAAGTAGGTCTTGGTGGTGGGGAAGGACAGGAACATCCTGCGGGGAGAAGCAGAGTGAGGGGTGGGGTTTGGGTCCGG
HBA1_7_CEX_T	GGTCAGCGCGTCGGCCACCTTCTTGCCGTGGCCCTTAACCTGGGCAGAGCCGTGGCTCAGGTCGAAGTGCGGGAAGTAGG
HBA1_8_CEX_T	ACAGCGCGTTGGGCATGTCGTCCACGTGCGCCACGGCGTTGGTCAGCGCGTCGGCCACCTTCTTGCCGTGGCCCTTAACC
HBA1_9_CEX_T	TCTCGCCCCTCGACCCAGATCGCTCCCGGCCCGCCGCTCACCTTGAAGTTGACCGGGTCCACCCGAAGCTTGTGCGCGTG
HBA1_10_CEX_T	AGGAAGGCGCCATCTCGCCCCTCGACCCAGATCGCTCCCGGCCCGCCGCTCACCTTGAAGTTGACCGGGTCCACCCGAAG
HBA1_11_CEX_T	GTGGGCGGCCAGGGTCACCAGCAGGCAGTGGCTTAGGAGCTGTGCAGAGAAGAGGGTCAGTGGGGCCGAGGGCCCAGGCC
HBA1_12_CEX_T	CACAGAAGCCAGGAACTTGTCCAGGGAGGCGTGCACCGCAGGGGTGAACTCGGCGGGGAGGTGGGCGGCCAGGGTCACCA
HBA1_13_CEX_T	GGCAAGAAGCATGGCCACCGAGGCTCCAGCTTAACGGTATTTGGAGGTCAGCACGGTGCTCACAGAAGCCAGGAACTTGT
HBA1_14_CEX_T	GAGGCCCAAGGGGCAAGAAGCATGGCCACCGAGGCTCCAGCTTAACGGTATTTGGAGGTCAGCACGGTGCTCACAGAAGC
HBA2_1_CEX_T	GGGAAGTAGGTCTTGGTGGTGGGGAAGGACAGGAACATCCTGCGGGGAGAAGCAGAGTGAGGGGTGGGGTTTGGGTCCGG
HBA2_2_CEX_T	GGGAAGGACAGGAACATCCTGCGGGGAGAAGCAGAGTGAGGGGTGGGGTTTGGGTCCGGGGCCAGGACGGTTGAGGGTGG
HBA2_3_CEX_T	GGTCAGCGCGTCGGCCACCTTCTTGCCGTGGCCCTTAACCTGGGCAGAGCCGTGGCTCAGGTCGAAGTGCGGGAAGTAGG
HBA2_4_CEX_T	ACAGCGCGTTGGGCATGTCGTCCACGTGCGCCACGGCGTTGGTCAGCGCGTCGGCCACCTTCTTGCCGTGGCCCTTAACC
HBA2_5_CEX_T	TCTCGCCCCTCGACCCAGATCGCTCCCGGCCCGCCGCTCACCTTGAAGTTGACCGGGTCCACCCGAAGCTTGTGCGCGTG
HBA2_6_CEX_T	AGGAAGGCGCCATCTCGCCCCTCGACCCAGATCGCTCCCGGCCCGCCGCTCACCTTGAAGTTGACCGGGTCCACCCGAAG
HBA2_7_CEX_T	GTGGGCGGCCAGGGTCACCAGCAGGCAGTGGCTTAGGAGCTGTGCAGAGAAGAGGGTCAGTGCGGCCCAGGCCCGCAGCC
HBA2_8_CEX_T	CACAGAAGCCAGGAACTTGTCCAGGGAGGCGTGCACCGCAGGGGTGAACTCGGCGGGGAGGTGGGCGGCCAGGGTCACCA
HBA2_9_CEX_T	GGAGGAACGGCTACCGAGGCTCCAGCTTAACGGTATTTGGAGGTCAGCACGGTGCTCACAGAAGCCAGGAACTTGTCCAG
HBA2_10_CEX_T	GAGGCCCAGCGGGCAGGAGGAACGGCTACCGAGGCTCCAGCTTAACGGTATTTGGAGGTCAGCACGGTGCTCACAGAAGC
HBB_1_CEX_T	GCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTT
HBB_2_CEX_T	GCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCT
HBB_3_CEX_T	TTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCAC
HBB_4_CEX_T	CACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGGTGAGTCTATGGGACGCTTGATGTTTTCTTTCCCCTTCTTT
HBB_5_CEX_T	GATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCT
HBB_6_CEX_T	TTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTT
HBB_7_CEX_T	ACTGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGA
HBB_8_CEX_T	GGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATA
HBB_9_CEX_T	GGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAG
HBB_10_CEX_T	TCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCATCTGACTCCTGAGGAGAAGTCTGCCGTTACT

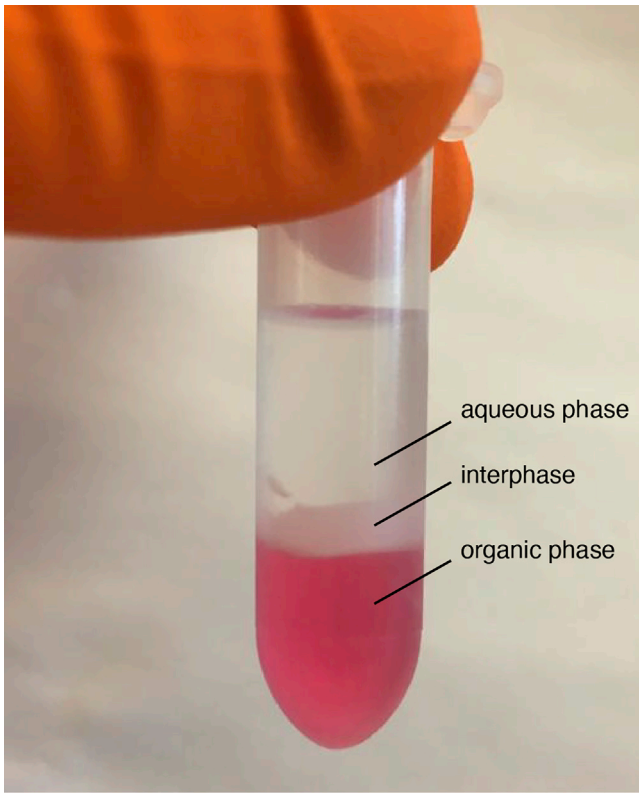

Figure 2. Phase separation during RNA purification protocol

Example of the three phases that are formed after the addition of chloroform and after centrifugation (step 4). The upper, aqueous phase contains RNA and should be carefully transferred in step 5.

Note: We recommend to isolate RNA from a maximum of 12 samples in parallel. Including more samples might hinder a fluent execution of the protocol and lead to a longer processing time per sample, possibly resulting in RNA degradation.

Note: This kit is a good compromise between low input volume and high mRNA yield as determined in the Extracellular RNA Quality Control Study (unpublished work). If more input volume is available, we recommend the QIAamp ccfDNA/RNA Kit (Cat# 55184, Qiagen) that can process up to 4 mL of biofluid. As also other kits exist, we recommend doing a comparative test in your lab using your samples, including spike-in RNA controls, to determine RNA purification performance parameters, such as yield, concentration, and precision.

Genomic DNA removal

Timing: ~30 min for 12 samples

This step describes the protocol for the removal of contaminating genomic DNA (gDNA) in total RNA samples extracted from biofluids using HL-dsDNAse (Cat# 70800-202, ArcticZymes) and 10× Reaction buffer (Cat# 66001, ArcticZymes).

12. Vortex the 10× Reaction buffer and flick the tube with HL-dsDNase enzyme, and spin both reagents down. Put the enzyme on a cooling block.

13. Create a reaction master mix containing HL-dsDNase and 10× reaction buffer for all samples on a cooling block. Per sample 1μL HL-dsDNase and 1.4μL 10× reaction buffer should be added in the master mix.
14. Flick the tubes with the mix and spin down.
15. Transfer 12 μL of each RNA sample to a 96-well plate.
16. Add 2 μL ERCC working solution c to each RNA sample in the 96-well plate.
17. Distribute 2.4 μL of the mix to each spike-in supplemented RNA sample in the 96-well plate.
18. Seal the 96-well plate using an adhesive PCR seal, flick the 96-well plate and spin down.
19. Visually inspect whether the sample wells contain an equal volume of reaction master mix and RNA.
20. Put the samples in a T100 Thermal Cycler and incubate for 10 min at 37°C, followed by 5 min at 58°C.
21. Immediately put the samples on a cooling unit upon the completion of the protocol.

Note: HL-dsDNase is especially developed to remove contaminating gDNA from RNA samples. The HL-dsDNase treatment of RNA does not affect the quantity of the RNA. The treatment however slightly fragments RNA. Therefore, multiple DNase treatments highly degrade RNA and are contra-indicated for downstream processes where RNA quality is essential for good performance (not for already highly fragmented exRNA). In addition, while both a single treatment and multiple treatments have an impact on qPCR measurements, conclusions of downstream RT-qPCR-based gene expression analyses do not change as long as all samples within the study are treated equally. Therefore, it is imperative that all samples undergo the same DNase treatment in order to be able to compare sample results.

Note: This step results in 16.4 μL DNase treated and spike-in added RNA, sufficient for ~2 library prep replicates (see step 3).

Pause point: Store the DNase treated RNA at −80°C and thaw the RNA samples on a cooling unit immediately before the start of the library prep. Avoid multiple freeze/thaw cycles.

mRNA: capture library preparation

Timing: ~4–5 days

Theoretical background of the library preparation protocol

This step explains how to convert total RNA into a sequencing-ready library of template molecules of known strand origin. The coding regions of the transcriptome are captured using the reagents provided in the Illumina TruSeq RNA Exome Library Prep kit (Cat# 20020189, Cat# 20020490, Cat# 20020183, Illumina). An overview of all steps is provided in Figure 3. The resulting library is suitable for subsequent cluster generation and sequencing. The protocol provided here is optimized to allow successful RNA sequencing of low-input biofluid samples containing fragmented RNA. In brief, modifications to the manufacturer's manual (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseq-rna-exome/truseq-rna-exome-reference-1000000039582-01.pdf) include a shorter incubation time in the fragmentation step (as RNA in biofluids is already degraded to some extent), an augmentation of the number of PCR cycles, and no pooling of samples during capture. A detailed step-by-step protocol is described below, preparation of the required consumables per step is summarized in Table 7. Throughout the mRNA capture preparation protocol the following plastics are used: 96-well PCR plates and seals (Cat# SP-0074, Cat# SP-0027, Cat# SP-0028, Cat# SP-0605, ThermoFisher Scientific) and RNase/DNase-free eight-tube strips and caps (Cat# SP-0085, ThermoFisher Scientific). The time needed for each step in the protocol depends on the experience of the researcher as well as the number of samples included in the library preparation. When performing the protocol for the first time, we recommend to foresee sufficient time. The timing provided here, is an indication for 48 samples performed by a skilled lab technician.

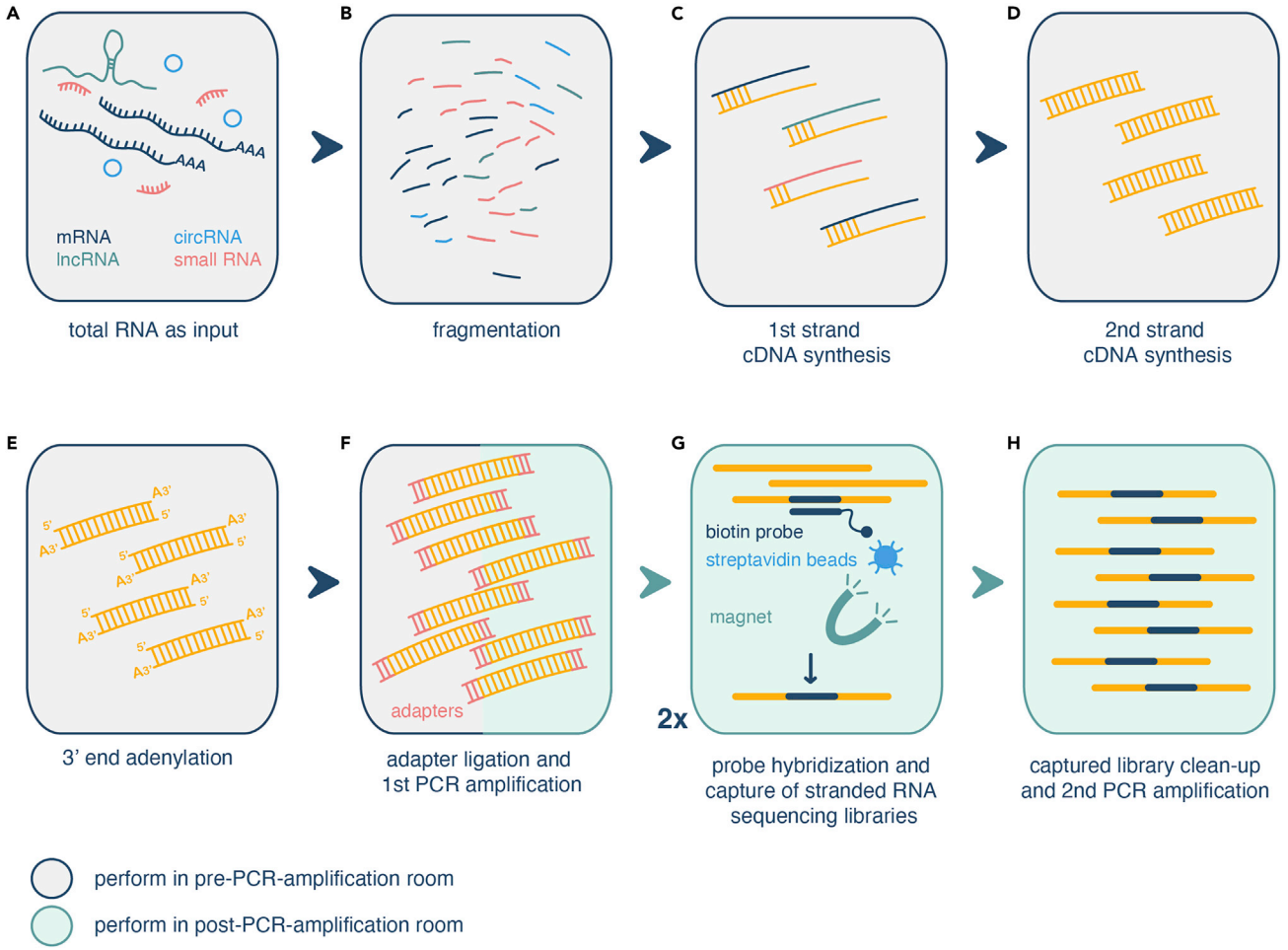

Figure 3. Schematic overview of the different steps in the TruSeq RNA Exome library preparation workflow

(A) Total RNA is used as input material for this library preparation.

(B) The RNA is fragmented using divalent cations under elevated temperature.

(C) cDNA is generated from the RNA fragments using random priming during first strand synthesis.

(D) The second strand synthesis removes the RNA template and synthesizes a replacement strand.

(E) During the 3' end adenylation step a single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction.

(F) During the ligation process the indexing adapters ligate to the ends of the ds cDNA, preparing them for hybridization onto a flow cell. This is followed by a PCR that is used to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library.

(G) The coding regions of the transcriptome are then captured from this library using sequence-specific, biotinylated probes to create the final library. This is followed by the first capture, using streptavidin beads to capture the biotinylated probes that are hybridized to the targeted regions of interest. Two heated wash procedures remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization. This second hybridization/capture is required to ensure high specificity of the captured regions.

(H) Finally, the captured library is purified using AMPure XP beads and a second PCR amplification is performed to amplify the enriched DNA library for sequencing. Note that all steps before the first PCR amplification should be performed in a separate, pre-PCR-amplification room to avoid contamination with PCR products.

Note: The Enrichment Wash Solution can be cloudy after vortexing.

Note: All steps of the library preparation protocol before the first PCR amplification should be performed in a separate, pre-PCR-amplification room to avoid contamination with PCR products.

Table 7. Preparation of consumables for mRNA capture library preparation

Protocol step(s) (Day)	Reagent	How to thaw	How to store upon thawing (during library preparation)
fragment RNA (day 1)	Elute, Prime, Fragment High Mix	room temperature	cooling unit
first strand cDNA synthesis (day 1)	SuperScript II Reverse Transcriptase	cooling unit[a]	cooling unit
	First Strand Synthesis Act D Mix	room temperature[a]	cooling unit
second strand cDNA synthesis (day 1)	Second Strand Marking Master Mix	room temperature	cooling unit
	Resuspension Buffer	room temperature	room temperature
ligate adapters (day 1)	Resuspension Buffer	room temperature	room temperature
first PCR amplification (day 1)	Resuspension Buffer	room temperature	room temperature
second strand cDNA synthesis (day 1)	AMPure XP beads	room temperature	room temperature
ligate adapters (day 1)	AMPure XP beads	room temperature	room temperature
first PCR amplification (day 1)	AMPure XP beads	room temperature	room temperature
adenylate 3′ ends (day 1)	A-Tailing Mix	room temperature	cooling unit
ligate adapters (day 1)	Ligation Mix	cooling unit[b]	cooling unit[b]
	RNA Adapter Indexes	room temperature	room temperature
	Stop Ligation Buffer	room temperature	room temperature
first PCR amplification (day 1)	PCR Primer Cocktail	cooling unit	cooling unit
	PCR Master Mix	cooling unit	cooling unit
first hybridization (day 2)	Capture Target Buffer 3	room temperature	room temperature
	Coding Exome Oligos	room temperature	room temperature
	Spike probes	room temperature	cooling unit
	1000× anti-CEX oligo pool	room temperature	cooling unit
first capture (day 2)	Streptavidin Magnetic Beads	room temperature	room temperature
	Enrichment Wash Solution	room temperature[c]	room temperature[c]
	Enrichment Elution Buffer 1	room temperature	room temperature
	2 N NaOH	room temperature	room temperature
	Elute Target Buffer 2	room temperature	room temperature
capture sample clean-up (day 3)	Resuspension Buffer	room temperature	room temperature
	AMPure XP beads	room temperature	room temperature
second hybridization (day 3)	Capture Target Buffer 3	room temperature	room temperature
	Coding Exome Oligos	room temperature	room temperature
	Spike probes	room temperature	cooling unit
	1000× anti-CEX oligo pool	room temperature	cooling unit
second capture (day 3)	Streptavidin Magnetic Beads	room temperature	room temperature
	Enrichment Wash Solution	room temperature[c]	room temperature[c]
	Enrichment Elution Buffer 1	room temperature	room temperature
	2 N NaOH	room temperature	room temperature
	Elute Target Buffer 2	room temperature	room temperature
second PCR clean-up (day 4)	Resuspension Buffer	room temperature	room temperature
	AMPure XP beads	room temperature	room temperature
second PCR amplification (day 4)	PCR Primer Cocktail	cooling unit	cooling unit
	Enhanced PCR Mix	cooling unit	cooling unit

Vortex and spin down all buffers, indices, oligos, and beads before use. Room temperature refers to the range between 18°C and 22°C. The cooling unit refers to the range between −18°C and −20°C.

[a]Flick and spin down the First Strand Synthesis Act D Mix and the SuperScript II Reverse Transcriptase before use.

[b]The Ligation Mix should be removed from −25°C to −15°C immediately before use, and returned to the freezer immediately after use.

[c]Upon removal from the freezer, the Enrichment Wash Solution is wrapped in aluminum foil.

Fragment RNA – day 1 (~20 min)

22. Pre-heat a T100 Thermal Cycler (Cat# 186-1096, Bio-Rad) according to the program indicated in Table 8.
23. A volume of 8.5 µL DNase-treated and spike-in supplemented RNA eluate is pipetted into a 96-well PCR plate on a cooling unit and mixed with 8.5 µL Elute, Prime, Fragment High Mix by pipetting up and down.

Table 8. Thermal cycler program to fragment RNA with lid temperature at 100°C and volume set to 17 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
pre-heat	94°C	∞	1 cycle
Incubation	94°C	2 min	1 cycle
Hold	4°C	∞	1 cycle

24. After sealing, the plate is shortly centrifuged (to remove droplets on the side and air bubbles) and continuously shaken on an Eppendorf ThermoMixer Comfort (Cat# 460-1112, Eppendorf) at 1600 rpm (4 × *g*) for 20 s and again shortly centrifuged at 280 × *g*.
25. Subsequently, the plate is placed on a pre-programmed T100 Thermal Cycler and incubated according to the program described in Table 8. When the thermal cycler reaches 4°C, the plate is removed, briefly centrifuged, and kept on a cooling unit.
26. First strand cDNA synthesis immediately follows RNA fragmentation.

Note: While it is common practice to end a program with an infinite cooling step at 4°C, we recommend taking the tubes as quickly as possible from the thermocycler, as this step consumes a lot of energy and wears the cycler.

First strand cDNA synthesis – day 1 (~1 h 15 min)

27. Pre-heat a thermal cycler according to the program indicated in Table 9.
28. Remove the adhesive seal from the plate.
29. Add 50 μl SuperScript II to the First Strand Synthesis Act D Mix tube if the whole kit is being used at once (48 reactions). Mix gently, but thoroughly, centrifuge briefly and keep the mix on a cooling block. Label the First Strand Synthesis Act D Mix tube to indicate that the SuperScript II has been added. If you are not using the entire content of the First Strand Synthesis Act D Mix tube, add Superscript II at a ratio of 1 μl SuperScript II for each 9 μl First Strand Synthesis Act D Mix. Mix by pipetting up and down. Store the mix on a cooling block.
30. Next, 8 μL of this mixture is added to the sample, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a ThermoMixer at 1600 rpm (4 × *g*) for 20 s.
31. Afterwards, the plate is shortly centrifuged at 280 × *g*, placed on a pre-programmed thermal cycler and incubated according to the program described in Table 9. When the thermal cycler reaches 4°C, the plate is removed, briefly centrifuged, and kept on a cooling unit.
32. Second strand cDNA synthesis immediately follows first strand cDNA synthesis.

Second strand cDNA synthesis – day 1 (~2 h)

33. Pre-heat a thermal cycler according to the program indicated in Table 10.
34. Remove the adhesive seal from the plate.

Table 9. Thermal cycler program to synthesize first strand cDNA with lid temperature at 100°C and volume set to 25 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
pre-heat	25°C	∞	1 cycle
Incubation	25°C	10 min	1 cycle
Incubation	42°C	15 min	1 cycle
Incubation	70°C	15 min	1 cycle
Hold	4°C	∞	1 cycle

Table 10. Thermal cycler program to synthesize second strand cDNA with lid temperature at 40°C and volume set to 50 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
pre-heat	16°C	∞	1 cycle
incubation	16°C	30 min	1 cycle

35. A volume of 20 μL Second Strand Marking Master Mix is mixed with 5 μL Resuspension Buffer on a cooling unit, by pipetting up and down.
36. Next, 25 μL of this mixture is added to the sample, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a ThermoMixer at 1600 rpm (4 × *g*) for 20 s.
37. Afterwards, the plate is shortly centrifuged at 280 × *g*, placed on a pre-programmed thermal cycler and incubated according to the program described in Table 10. The plate is then removed from the cycler, shortly centrifuged at 280 × *g* and placed on the bench for 1 min to bring it to room temperature (18°C – 22°C).
38. Vortex the AMPure XP beads (Cat# A63881, Beckman Coulter Life Sciences) until they are well dispersed.
39. Subsequently, 90 μL well-dispersed AMPure XP beads is added to the sample, followed by pipetting up and down, sealing the plate and continuously shaking it on a ThermoMixer at 1800 rpm (5 × *g*) for 2 min.
40. The plate is then incubated for 5 min at room temperature (18°C – 22°C) and shortly centrifuged at 280 × *g*. Remove the adhesive seal and place the plate on a magnetic stand for 5 min at room temperature (18°C – 22°C) to make sure that all of the beads are bound on the side of the wells.
41. All supernatant is removed and 200 μL freshly prepared 80% ethanol is added without disturbing the beads.
42. The plate is incubated for 30 s and again all supernatant removed. This washing step is repeated for a total of two 80% ethanol washes.
43. Then, the plate is dried for 5 min at room temperature (18°C – 22°C) and 20 μL Resuspension Buffer is pipetted onto the beads. To resuspend the beads, the plate is removed from the magnetic stand and the sample is pipetted up and down.
44. The plate is shaken on a ThermoMixer at 1800 rpm (5 × *g*) for 2 min, incubated on the bench for 2 min at room temperature (18°C – 22°C) and shortly centrifuged at 280 × *g*.
45. Finally, the plate is placed on the magnetic stand for 5 min at room temperature (18°C – 22°C) and 17.5 μL supernatant (i.e., ds cDNA) is transferred to a new plate.

Pause point: The library prep protocol can be safely paused here. If you are stopping, seal the plate and store at −25°C to −15°C for up to 7 days. Longer storage is likely possible but should be verified by the user.

Adenylate 3′ ends – day 1 (~45 min)

46. Pre-heat a thermal cycler according to the program indicated in Table 11.
47. Remove the adhesive seal from the plate.
48. 3′ ends are adenylated by adding 12.5 μL A-Tailing Mix to the sample, followed by pipetting up and down, and shaking the sealed plate on a ThermoMixer at 1800 rpm (5 × *g*) for 2 min.
49. After shortly centrifuging the plate at 280 × *g*, it is placed on a pre-programmed thermal cycler and incubated according to the program described in Table 11. Immediately put the plate on a cooling unit when the program is finished for 1 min.

Table 11. Thermal cycler program to adenylate 3′ ends with lid temperature at 100°C and volume set to 30 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
pre-heat	37°C	∞	1 cycle
Incubation	37°C	30 min	1 cycle
Incubation	70°C	5 min	1 cycle

Ligate adapters – day 1 (~1 h 45 min)

50. Pre-heat a thermal cycler according to the program indicated in Table 12.
51. Remove the adhesive seal from the plate.
52. A volume of 2.5 μL Resuspension Buffer is mixed with 2.5 μL Ligation Mix on a cooling unit, by pipetting up and down.
53. Next, 5 μL of this mixture is added to the sample, as well as 2.5 μL RNA Adapter Index, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 2 min.

Note: A unique index should be added to each sample.

54. Afterwards, the plate is shortly centrifuged at 280 × *g*, placed on a pre-programmed thermal cycler and incubated according to the program described in Table 12.
55. Upon removing the plate from the cycler, 5 μL Stop Ligation Buffer is added, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 2 min. The plate is then centrifuged at 280 × *g* for 1 min.
56. Subsequently, 42 μL well-dispersed AMPure XP beads is added to the sample, followed by pipetting up and down, sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 2 min.
57. The plate is then incubated for 5 min at room temperature (18°C – 22°C), shortly centrifuged at 280 × *g* and placed on a magnetic stand for 2 min at room temperature (18°C – 22°C) to make sure that all of the beads are bound on the side of the wells.
58. All supernatant is removed and 200 μL freshly prepared 80% ethanol is added without disturbing the beads.
59. The plate is incubated for 30 s and again all supernatant removed. This washing step is repeated for a total of two 80% ethanol washes.
60. Then, the plate is dried for 5 min at room temperature (18°C–22°C) and 22.5 μL Resuspension Buffer is pipetted onto the beads. To resuspend the beads, the plate is removed from the magnetic stand and the sample is pipetted up and down.
61. The plate is shaken on a Thermomixer at 1800 rpm (5 × *g*) for 2 min, incubated on the bench for 2 min at room temperature (18°C–22°C) and shortly centrifuged at 280 × *g*.
62. Finally, the plate is placed on the magnetic stand for 2 min at room temperature (18°C – 22°C) and 20 μL supernatant is transferred to a new plate.

Pause point: The library prep protocol can be safely paused here. If you are stopping, seal the plate and store at −25°C to −15°C for up to 7 days. Longer storage is likely possible but should be verified by the user.

Table 12. Thermal cycler program to ligate adapters with lid temperature at 100°C and volume set to 38 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
pre-heat	30°C	∞	1 cycle
Incubation	30°C	10 min	1 cycle

First PCR amplification – day 1 (~2 h 10 min)

63. Pre-heat a thermal cycler according to the program indicated in Table 13.
64. A volume of 5 μL PCR Primer Cocktail (Cat# 20020189, Illumina) is mixed with 25 μL thawed PCR Master Mix (Cat# 20020189, Illumina) on a cooling unit, by pipetting up and down.
65. Next, 30 μL of this mixture is added to the sample, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1600 rpm (4 × *g*) for 20 s.
66. Afterwards, the plate is shortly centrifuged at 280 × *g*, placed on a pre-programmed thermal cycler and incubated according to the program described in Table 13.
67. Upon removing the plate from the cycler, 50 μL well-dispersed AMPure XP beads is added to the sample, followed by pipetting up and down, sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 2 min.
68. The plate is then incubated for 5 min at room temperature (18°C – 22°C), shortly centrifuged at 280 × *g*. The seal is removed, and the plate placed on a magnetic stand for 2 min at room temperature to make sure that all of the beads are bound on the side of the wells.
69. All supernatant is removed and 200 μL freshly prepared 80% ethanol is added without disturbing the beads.
70. The plate is incubated for 30 s and again all supernatant removed. This washing step is repeated for a total of two 80% ethanol washes.
71. Then, the plate is dried for 5 min at room temperature (18°C–22°C) and 14.5 μL Resuspension Buffer is pipetted onto the beads.
72. To resuspend the beads, the plate is removed from the magnetic stand and the sample is pipetted up and down. The plate is shaken on a Thermomixer at 1800 rpm (5 × *g*) for 2 min, incubated on the bench for 2 min at room temperature (18°C–22°C) and shortly centrifuged at 280 × *g*.
73. Finally, the plate is placed on the magnetic stand for 2 min at room temperature (18°C–22°C) and 12 μL supernatant is transferred to a new plate.

Pause point: The library prep protocol can be safely paused here. If you are stopping, seal the plate and store at −25°C to −15°C for up to 7 days. Longer storage is likely possible but should be verified by the user.

Validate library – day 1 (~5 h)

74. Libraries are validated using the Agilent HS Small Fragment Kit (Cat# DNF-477-0500), according to the manufacturer's Kit Guide (https://www.agilent.com/cs/library/usermanuals/public/quick-guide-dnf-477-hs-small-fragment-kit-SD-AT000136.pdf). Dilute the library to a concentration between 0.1 ng/μl and 5 ng/μl for use with the HS Small Fragment kit of the Fragment Analyzer. Only use 1 μL of the final library to create a dilution with 1 μL RNase free water. Load 2 μl of diluted library on the Fragment Analyzer. Check the size and purity of the sample.

Table 13. Thermal cycler program for the first PCR amplification with lid temperature at 100°C and volume set to 50 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
pre-heat	98°C	∞	1 cycle
Incubation	98°C	30 s	1 cycle
Denaturation	98°C	10 s	15 cycles
Annealing	60°C	30 s	
Extension	72°C	30 s	
Incubation	72°C	5 min	1 cycle
Hold	4°C	∞	1 cycle

The final product should be a smear from 160 bp to 700 bp, with a peak at approximately 260 bp. Figure 3 shows an example of a good-quality library. **Troubleshooting 3**

First hybridization – day 2 (~2 h 30 min)

75. Pre-heat a thermal cycler according to the program indicated in Table 14.
76. Thoroughly vortex the Capture Target Buffer 3 tube until the solution is completely re-suspended. Visually make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex the Capture Target Buffer 3 tube until it is clear. It is very important that the Capture Target Buffer 3 is clear before starting the first hybridization. Keep the Capture Target Buffer 3 at room temperature (18°C – 22°C) to avoid the formation of crystals.
77. 12.5 μL Capture Target Buffer 3 is mixed with 1.25 μL Coding Exome Oligos, 0.625 μL spike probes and 1.25 μL 1000× anti-CEX oligo pool by pipetting up and down. Keep this mix at room temperature (18°C – 22°C).
78. Next, 15.625 μL of this mixture is added to 11 μL sample, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1200 rpm (2 × *g*) for 1 min.
79. Afterwards, the plate is shortly centrifuged at 280 × *g*, placed on a pre-programmed thermal cycler and incubated according to the program described in Table 14.
80. When the incubation program is finished, the plate is immediately removed from the thermal cycler. First capture immediately follows first hybridization.

Note: In the original TruSeq RNA Exome protocol provided by Illumina, a pooling step before hybridization is described to combine multiple libraries with different indexes into a single pool before enrichment, allowing a high-throughput preparation of your samples. We do not recommend this for biofluid samples, as pooling before capture might impede equimolar pooling of the individual samples in step 133.

Table 14. Thermal cycler program for hybridization with lid temperature at 100°C and volume set to 25 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
pre-heat	95°C	∞	1 cycle
Incubation	95°C	10 min	1 cycle
Incubation	94°C	1 min	1 cycle
Incubation	92°C	1 min	1 cycle
Incubation	90°C	1 min	1 cycle
Incubation	88°C	1 min	1 cycle
Incubation	86°C	1 min	1 cycle
Incubation	84°C	1 min	1 cycle
Incubation	82°C	1 min	1 cycle
Incubation	80°C	1 min	1 cycle
Incubation	78°C	1 min	1 cycle
Incubation	76°C	1 min	1 cycle
Incubation	74°C	1 min	1 cycle
Incubation	72°C	1 min	1 cycle
Incubation	70°C	1 min	1 cycle
Incubation	68°C	1 min	1 cycle
Incubation	66°C	1 min	1 cycle
Incubation	64°C	1 min	1 cycle
Incubation	62°C	1 min	1 cycle
Incubation	60°C	1 min	1 cycle
Incubation	58°C	90 min	1 cycle
Hold	58°C	∞	1 cycle

First capture – day 2 (~2 h 30 min)

81. Pre-heat a thermal cycler according to the program indicated in Table 15.
82. Centrifuge the plate at 280 × *g* for 1 min and remove the adhesive seal. Take care when removing the seal to avoid spilling the contents of the wells.
83. Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed.
84. 62.5 μL well-dispersed Streptavidin Magnetic Beads is added, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1200 rpm (2 × *g*) for 5 min.
85. The plate is then incubated for 25 min at room temperature (18°C – 22°C), shortly centrifuged at 280 × *g* and placed on a magnetic stand for 2 min at room temperature (18°C – 22°C) to make sure that all of the beads are bound on the side of the wells. All supernatant is removed.
86. Subsequently, 50 μL Enrichment Wash Solution is added, followed by centrifuging the plate at 280 × *g* for 10 s, and pipetting up and down to resuspend the beads. Use a volume of 45 μL to pipet up and down in order to avoid air bubbles.
87. Next, the plate is sealed and thoroughly mixed by continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 4 min, and placed on a pre-programmed thermal cycler and incubated according to the program described in Table 15.
88. Upon incubation, the plate is immediately shortly centrifuged at 280 × *g*. The seal is removed and the plate is placed on a magnetic stand for 2 min.
89. Discard all supernatant and remove the plate from the magnetic stand. This wash step is repeated one more time, for a total of two washes (repeat from step 86 onwards).
90. Upon washing, elution pre-mix is made by mixing 11.4 μL Enrichment Elution Buffer 1 and 0.6 μL 2 N NaOH, followed by vortexing the mixture.
91. Next, 11 μL of this elution pre-mix is added to the sample, followed by centrifuging at 280 × *g* for 10 s, pipetting up and down (with a volume lower than 11 μL to avoid bubbles), and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 2 min.
92. The plate is then incubated for 2 min at room temperature (18°C – 22°C), shortly centrifuged at 280 × *g* and placed on a magnetic stand for 2 min at room temperature (18°C – 22°C) to make sure that all of the beads are bound on the side of the wells.
93. Then, 9 μL supernatant is transferred to a new plate, pre-filled with 1.7 μL Elute Target Buffer 2, followed by pipetting up and down, and incubating the plate on a Thermomixer at 1200 rpm (2 × *g*) for 1 min to neutralize the elution.
94. Afterwards, the plate is shortly centrifuged at 280 × *g*.

Pause point: The library prep protocol can be safely paused here. If you are stopping, seal the plate and store at −25°C to −15°C for up to 7 days. Longer storage is likely possible, but should be verified by the user.

Second hybridization – day 3 (~2 h 30 min)

95. Pre-heat a thermal cycler according to the program indicated in Table 14.
96. During the second hybridization, anti-coding exome oligos (anti-CEX oligo pool; Integrated DNA Technologies) are used to ensure that no globin is captured. More precisely, 12.5 μL

Table 15. Thermal cycler program for the first capture with lid temperature at 100°C and volume set to 50 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
pre-heat	50°C	∞	1 cycle
Incubation	50°C	20 min	1 cycle
Hold	50°C	∞	1 cycle

Capture Target Buffer 3 is mixed with 1.25 μL Coding Exome Oligos, 0.625 μL spike probes and 1.25 μL 1000× anti-CEX pool (Table 6) by pipetting up and down.

97. Next, 15.625 μL of this mixture is added to 10.7 μL sample, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1200 rpm (2 × *g*) for 1 min.
98. Afterwards, the plate is shortly centrifuged at 280 v *g*, placed on a pre-programmed thermal cycler and incubated according to the program described in Table 14. When the incubation program is finished, the plate is immediately removed from the thermal cycler.
99. Second capture immediately follows second hybridization.

Second capture – day 3 (~2 h 30 min)

100. Pre-heat a thermal cycler according to the program indicated in Table 15.
101. Upon centrifuging the plate at 280 × *g* for 1 min, 62.5 μL well-dispersed Streptavidin Magnetic Beads is added, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1200 rpm (2 × *g*) for 5 min.
102. The plate is then incubated for 25 min at room temperature (18°C – 22°C) and shortly centrifuged at 280 × *g*. The seal is removed, and the plate is placed on a magnetic stand for 2 min at room temperature (18°C – 22°C) to make sure that all of the beads are bound on the side of the wells. All supernatant is removed.
103. Subsequently, 50 μL Enrichment Wash Solution is added, followed by centrifuging the plate at 280 × *g* for 10 s, and pipetting up and down to resuspend the beads.
104. Next, the plate is sealed and thoroughly mixed by continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 4 min and placed on a pre-programmed thermal cycler and incubated according to the program described in Table 15.
105. Upon incubation, the plate is immediately shortly centrifuged at 280 × *g*. The seal is removed and the plate is placed on a magnetic stand for 2 min. All supernatant is removed and the plate removed from the magnetic stand. This wash step is repeated one more time, for a total of two washes (repeat from step 103 onwards).
106. Upon washing, elution pre-mix is made by mixing 11.4 μL Enrichment Elution Buffer 1 and 0.6 μL 2 N NaOH, followed by vortexing the mixture.
107. Next, 11 μL of this elution pre-mix is added to the sample, followed by centrifuging at 280 × *g* for 10 s, pipetting up and down (with a volume lower than 11 μL to avoid bubbles), and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 2 min.
108. The plate is then incubated for 2 min at room temperature (18°C–22°C), shortly centrifuged at 280 × *g* and placed on a magnetic stand for 2 min at room temperature (18°C–22°C) to make sure that all of the beads are bound on the side of the wells.
109. Then, 9 μL supernatant is transferred to a new plate, pre-filled with 1.7 μL Elute Target Buffer 2, followed by pipetting up and down, and incubating the plate on a Thermomixer at 1800 rpm (5 × *g*) for 1 min to neutralize the elution.
110. Afterwards, the plate is shortly centrifuged at 280 × *g*.
111. Capture sample clean up immediately follows second capture.

Capture sample clean-up – day 3 (~1 h)

112. Subsequently, 20 μL well-dispersed AMPure XP beads is added to the sample, followed by pipetting up and down, sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 2 min.
113. The plate is then incubated for 5 min at room temperature (18°C – 22°C), shortly centrifuged at 280 × *g* and placed on a magnetic stand for 2 min at room temperature (18°C – 22°C) to make sure that all of the beads are bound on the side of the wells.

114. All supernatant is removed and 200 µL freshly prepared 80% ethanol is added without disturbing the beads. The plate is incubated for 30 s and again all supernatant removed. This washing step is repeated for a total of two 80% ethanol washes.
115. Then, the plate is dried for 5 min at room temperature (18°C–22°C) and 27.5 µL Resuspension Buffer is pipetted onto the beads.
116. To resuspend the beads, the plate is removed from the magnetic stand and the sample is pipetted up and down.
117. The plate is shaken on a Thermomixer at 1800 rpm (5 × *g*) for 1 min, incubated on the bench for 2 min at room temperature (18°C–22°C) and shortly centrifuged at 280 × *g*.
118. Finally, the plate is placed on the magnetic stand for 2 min at room temperature (18°C–22°C) and 25 µL supernatant is transferred to a new plate.

Pause point: The library prep protocol can be safely paused here. If you are stopping, seal the plate and store at −25°C to −15°C for up to 7 days. Longer storage is likely possible but should be verified by the user.

Second PCR amplification – day 4 (~1 h)

119. Pre-heat a thermal cycler according to the program indicated in Table 16.
120. A volume of 5 µL PCR Primer Cocktail (Cat# 20020189, Illumina) is mixed with 20 µL Enhanced PCR Mix (Cat# 20020189, Illumina) on a cooling unit, by pipetting up and down.
121. Next, 25 µL of this mixture is added to the sample, followed by pipetting up and down, and thoroughly mixing by sealing the plate and continuously shaking it on a Thermomixer at 1200 rpm (2 × *g*) for 1 min.
122. Afterwards, the plate is shortly centrifuged at 280 × *g*, placed on a pre-programmed thermal cycler and incubated according to the program described in Table 16.
123. Upon incubation, the plate is shortly centrifuged at 280 × *g*.

Second PCR clean-up – day 4 (~1 h)

124. 90 µL well-dispersed AMPure XP beads is added to the sample, followed by pipetting up and down, sealing the plate and continuously shaking it on a Thermomixer at 1800 rpm (5 × *g*) for 1 min.
125. The plate is then incubated for 5 min at room temperature (18°C – 22°C) and shortly centrifuged at 280 × *g*. The seal is removed and the plate is placed on a magnetic stand for 2 min at room temperature (18°C – 22°C) to make sure that all of the beads are bound on the side of the wells.
126. All supernatant is removed and 200 µL freshly prepared 80% ethanol is added without disturbing the beads.
127. The plate is incubated for 30 s and again all supernatant removed. This washing step is repeated for a total of two 80% ethanol washes.

Table 16. Thermal cycler program for the second PCR amplification with lid temperature at 100°C and volume set to 50 µL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
pre-heat	98°C	∞	1 cycle
Incubation	98°C	30 s	1 cycle
Denaturation	98°C	10 s	14 cycles
Annealing	60°C	30 s	
Extension	72°C	30 s	
Incubation	72°C	5 min	1 cycle
Hold	10°C	∞	1 cycle

128. Then, the plate is dried for 5 min at room temperature (18°C–22°C) and 32 μL Resuspension Buffer is pipetted onto the beads.
129. To resuspend the beads, the plate is removed from the magnetic stand and the sample is pipetted up and down. The plate is shaken on a Thermomixer at 1800 rpm (5 × *g*) for 1 min, incubated on the bench for 2 min at room temperature (18°C–22°C) and shortly centrifuged at 280 × *g*.
130. The plate is placed on the magnetic stand for 2 min at room temperature (18°C–22°C) and 30 μL supernatant is transferred to a new plate.

Pause point: The library prep protocol can be safely paused here. If you are stopping, seal the plate and store at −25°C to −15°C for up to 7 days. Longer storage is likely possible, but should be verified by the user.

Validate library – day 4/day 5 (~5 h)

131. Libraries are validated using the HS Small Fragment Kit (Cat# DNF-477-0500, Agilent), according to the manufacturer's Kit Guide (https://www.agilent.com/cs/library/usermanuals/public/quick-guide-dnf-477-hs-small-fragment-kit-SD-AT000136.pdf). Library concentrations were determined using Fragment Analyzer software for smear analysis in the 160 bp to 700 bp range. Dilute the library to a concentration between 0.1 ng/μl and 5 ng/μl for use with the HS Small Fragment kit of the Fragment Analyzer. Use 1 μl of the library and add 1 μL of RNase free water. Load 2 μl of diluted library on the Fragment Analyzer. Check the size and purity of the sample. The final product should be a smear from 160 bp to 700 bp with a peak at approximately 400 bp. **Troubleshooting 4**

Library pooling and quantification – day 4/day 5 (~7 h 45 min)

132. Quantify the libraries by qPCR using the KAPA Library Quantification Kit, according to the manufacturer's guide (https://pim-eservices.roche.com/eLD/api/downloads/ca670ceb-fb38-eb11-0291-005056a71a5d?countryIsoCode=pi) and calculate library molarity.
133. Dilute each library to 0.5 nM (or the highest possible concentration, depending on the biofluid) and pool equimolar concentrations of each library in a microfuge tube.
134. Libraries are now ready for sequencing.

Sequence libraries

135. Sequence the libraries on an Illumina instrument (NextSeq 500, HiSeq 2/3000 or NovaSeq 6000). If using the NextSeq 500 platform for a pool obtained from platelet-free plasma, we recommend to load a 1.6 pM library with 5% PhiX for paired-end sequencing using a NextSeq 500/550 HO kit v2.5 150 cycles (Cat# 20024907, Illumina). **Troubleshooting 5**

EXPECTED OUTCOMES

RNA purification

The concentration of the extracellular RNA obtained from biofluids is often too low to be measurable with Nanodrop or Qubit. As the RNA is highly fragmented in biofluids, it is of no use to evaluate the RNA integrity using e.g., an Agilent Bioanalyzer or Fragment Analyzer. We therefore volumetrically standardize and use the same RNA eluate volume (from the same biofluid input volume) into the reaction.

Genomic DNA removal

Since gDNA removal is crucially important for the analysis of extracellular mRNAs (see Verwilt et al, (Verwilt et al., 2020)), we recommend to experimentally verify the (almost complete) absence of contaminating gDNA in your samples, especially if it is the first time you perform gDNA removal in your lab. RT-qPCR using any two of four DNA specific assays provided in Table 17 can be

Table 17. DNA specific assays to assess the genomic DNA contamination in RNA samples

Gene Locus	Forward primer	Reverse primer
NEUROD1	CTTCTGCCGCCTGAAAGG	CCTGGAACCACGTGACCTG
XRCC3	CTTGATTCTTTCTAGCCTTGG	GGTTGACACTTTGATGGATAC
PLAT	GCGTGGCTTCTCTCTGATCC	GAGCTCTGGCTTTTGCATCTG
MTHFD2	GTTCCCTTACTGGGTGGTGCTA	AGTTACTGCTTCAACCACGTGATC

performed directly on the RNA samples (without making cDNA, i.e. RT- controls) (both before and after gDNA removal), as previously described(Van Peer et al., 2012). A complete to almost complete eradication of the DNA specific signal should be observed with all assays.

In addition, you can also verify the absence of DNA contamination in the final sequencing data, as described (Verwilt et al., 2020).

mRNA capture library preparation

After step 74, you should expect to generate cDNA libraries before capture such as the one presented in Figure 4, with a smear from 160 bp to 700 bp and a peak at approximately 260 bp.

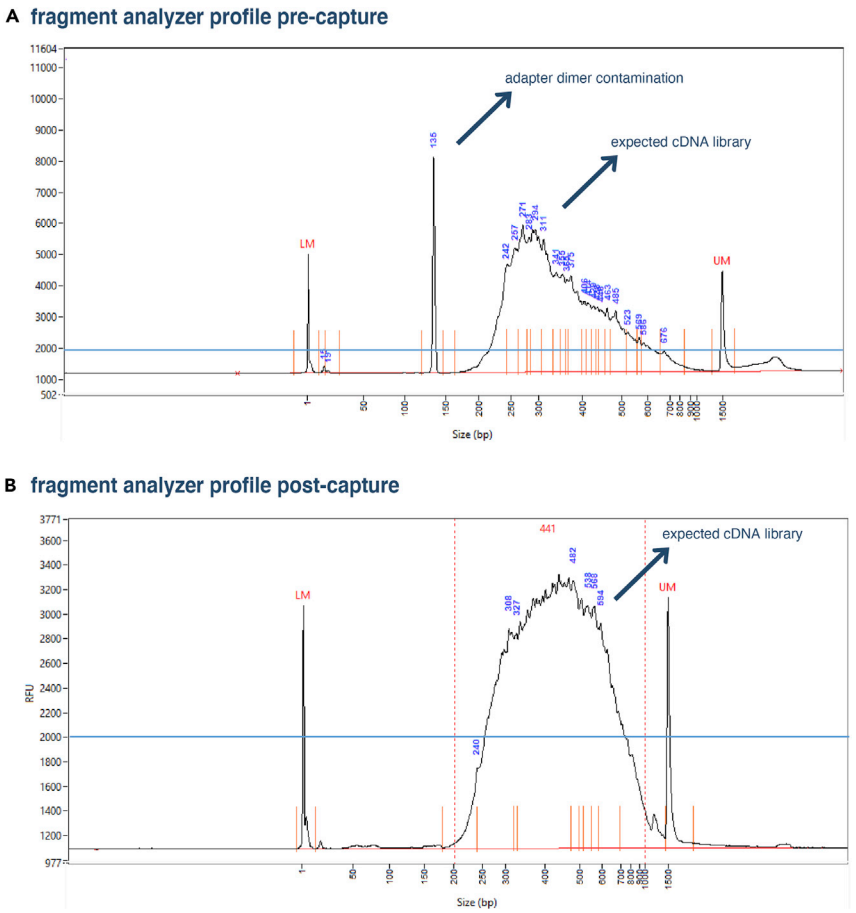

Figure 4. Expected fragment analyzer profiles

(A) Fragment analyzer profile of a good-quality cDNA library obtained from platelet-free plasma, before the first hybridization step. A smear from 160 bp to 700 bp with a peak around 260 bp is expected. Adapter dimers can be present as a sharp peak around 120 bp.

(B) Fragment analyzer profile of a good quality cDNA library obtained from platelet-free plasma, after the second PCR clean-up. A band around 400 bp is expected. Adapter dimers are not present anymore.

After step 131, you should expect to generate the final cDNA libraries after capture such as the one presented in Figure 4, with a smear from 160 bp to 700 bp and a peak at approximately 400 bp.

QUANTIFICATION AND STATISTICAL ANALYSIS

Processing TruSeq RNA Exome sequencing data

When obtaining sequencing results, a thorough inspection of QC metrics is key before initiating other analysis. Illumina's BaseSpace (basespace.illumina.com) contains useful data presented in the "Per Read and Per Lane Metrics" tables, including sequencing reads, error rate, percentage >=Q30, density (K/mm^2), cluster PF(%). We generally aim for 10M reads per sample (if the purpose of the analysis is gene level quantification and gene set enrichment analysis) and 20M reads per sample (if the purpose of the analysis is an in-depth inspection of genes with low abundance). Verify if the percentage of reads that are aligned to PhiX control V3 (Cat# FC-110-3001, Illumina) are similar to the loaded PhiX concentration. The error rate reflects the percentage of bases called incorrectly at any cycle. It is calculated from the reads that are aligned to PhiX. Percentage >=Q30 should be preferably above 90% and reads passing filter (PF) above 85%. More in-depth QC assessment should be performed using FastQC (v0.11.5).

Several processing pipelines can be used to process TruSeq RNA Exome sequencing data. The computational pipeline used in our lab is available on Github (https://github.com/OncoRNALab/RNAexome). We recommend the following steps for an alignment-based analysis:

1. Read quality can be assessed by running FastQC (v0.11.5) on the FASTQ files. Reads shorter than 35 nucleotides and reads where fewer than 80% of bases have a (phred) score above 20 should be removed.
2. The reads can be mapped with STAR (v2.6.0)(Dobin et al., 2013). Mapped reads are annotated by matching genomic coordinates of each read with genomic locations of mRNAs (obtained from UCSC GRCh38/hg38 and Ensembl v91) or by matching the spike-in sequences.
3. Duplicate removal with Picard (v2.18.5).
4. Quantification with HTSeq (v0.9.1) of the PCR deduplicated reads.

We recommend to apply a cut-off for filtering out noisy genes. We set this threshold to the number of counts where at least 95% of the single positive replicate values are removed as previously proposed (Mestdagh et al., 2014).

Normalization strategies and calculation of endogenous mRNA concentration based on spike-in controls

A biofluid volume-based normalization strategy can be applied by dividing the number of RNA reads consumed by the endogenous transcripts by the sum of the Sequin reads. The spike-normalized data represent relative abundance values of mRNA molecules proportional to the biofluid input volume. Whether normalization based on endogenous RNA or spike-in RNA is more appropriate depends on the question asked, on the biofluid and on the condition or disease. We recommend evaluating both approaches and determine the most suitable method in each particular experiment design.

The absolute mass of endogenous mRNA present in 1 mL of a biofluid sample is estimated based on the read counts for a specific spike-in RNA. For example, R1_11 is one of the highest concentrated Sequin spikes with a concentration of 1933.594 attomol/µL (stock concentration) and is expected to be detected in every sample. R1_11 has a length of 785 nucleotides. For this example, we assume the RNA purification is performed on 200 µL of a platelet-rich plasma (PRP) sample and 2 µL of Sequin working solution c is added prior to the RNA purification, as recommended in our protocol.

A (Sequin spiked) PRP sample of 200 µL contains 29.75 e-22 mol of R1_11 (= 1933.594 attomol/µL × 2 µL × 1/1 300 000). The molecular weight of single stranded RNA (ssRNA) can be calculated based

on the following formula: *molecular weight of ssRNA (g/mol) = (length of ssRNA (nt) × 321.47 g/mol/nt) + 18.02 g/mol.* The molecular weight of R1_11 is thus 252 371.97 *g*/mol. A PRP sample of 200 μL contains 7.51 e-16 *g* R1_11.

The mass of endogenous mRNA in 200 μL of the biofluid sample is estimated by multiplying the mass of R1_11 in 200 μL of the biofluid sample by the ratio of the total reads mapping to mRNAs and the reads mapping to R1_11. For example, a PRP sample with 419 731 total mRNA read counts and 56 reads mapping to R1_11, contains 0.0056 ng endogenous mRNA. In conclusion, the endogenous mRNA concentration of this PRP sample can be estimated as 0.028 ng/mL.

> ***Note:*** Here, we focus on the analysis of linear mRNA. CircRNAs can also be detected with this protocol through the capture of exons that are incorporated in the circRNAs, followed by identification of the characteristic backsplice junction. To analyze the circRNA content, dedicated bio-informatics should be used, as described in Hulstaert et al(Hulstaert et al., 2020).

LIMITATIONS

This library preparation protocol focuses on the human mRNA and circRNA transcriptome. This protocol does not allow profiling of small RNAs, long non-coding RNAs and other RNA biotypes. Of note, the exome probe set from the TruSeq RNA Exome kit could be replaced by a custom probe set against human long non-coding RNAs (as done by Annelien Morlion, Celine Everaert et al. (unpublished work) or a commercial probe set for the murine exome).

The available number of indexes limits the number of samples that can be analyzed in a single experiment. When using TruSeq RNA Single Indexes Set A and B (Cat# 20020492 and Cat# 20020493, Illumina) 24 samples can be sequenced in a single experiment. When using the TruSeq RNA CD Index Plate (Cat# 20019792, Illumina), up to 96 samples can be sequenced together.

High-throughput sequencing platforms, including the Illumina platform used here, require PCR amplification during library preparation to obtain sufficient cDNA molecules for sequencing. In the optimized workflow for biofluids the number of PCR cycles is increased, as biofluid samples are considered as low-input samples. During this process, PCR duplicates (reads that are made from the same original cDNA molecule via PCR) will arise and these will impact the data-analysis. Duplicate removal, using computation tools such as Picard (http://broadinstitute.github.io/picard/) or BBMap's Clumpify utility (sourceforge.net/projects/bbmap/), should be performed when pre-processing RNA sequencing data from biofluids. The expected percentage of duplicated reads depends on the duplicate removal tool and the analyzed biofluid type, but is generally >90% for plasma.

TROUBLESHOOTING

Problem 1

You do not obtain a clear phase separation (as shown in Figure 2) after step 5 of the RNA isolation.

Potential solution

Make sure that the centrifuge does not heat above 10°C during centrifugation. If no phase separation is obtained, you can put the tube(s) with a troubled phase separation in the fridge for 8–12 h, repeat the centrifugation step (15 min at 12 000 *g* at 4°C) and continue from step 5 of the RNA isolation protocol onwards. Alternatively, if enough volume is left of the biofluid sample to repeat the RNA extraction, the RNA isolation can be repeated from step 1 onwards.

Problem 2

No eluate is obtained in step 10 of the RNA isolation.

Potential solution

A clogged RNA isolation column can be observed when purifying RNA from viscous biofluids (e.g., seminal plasma). An additional centrifugation step of 5 min at full speed can be added.

Problem 3

If no peak around 260 bp is observed on fragment analyzer (before the first hybridization step, step 74, Figure 4), then the RNA that was used as input for the library preparation was highly degraded or the library preparation failed due to human handling error (e.g., accidently discarding supernatant when it should be kept).

Potential solution

Carefully control the pre-analytical variables from the biofluid collection onwards and optimize these to prevent RNA degradation. RNA sequencing results of biofluids can be impacted by a broad range of pre-analytical variables, including collection tube, degree of hemolysis, time between blood draw and plasma preparation, RNA isolation kit, among others. The implementation of standardized and uniform methods for sample collection, processing, and profiling is of uttermost importance to enable a correct interpretation of sequencing results obtained by the workflow presented here.

Problem 4

If adapter-dimers are still visible as a peak around 120 bp on fragment analyzer after the second PCR clean-up (step 130, Figure 4), these contaminating adapter-dimers might cause issues with exclusion-amplification clustering chemistry on Illumina platforms.

Potential solution

Consider repeating the final bead clean-up (step 124–130) if the adapter-dimer peak (around 120 bp) is higher than the actual cDNA library (peak around 400 bp).

Problem 5

When sequencing samples with a broad complexity range, it might be impossible to prepare an equimolar pool including all samples (step 132).

Potential solution

Performing a pre-run (e.g., a mid-output run) can help you to divide samples over 2 separate high-output runs, based on the total reads obtained per sample.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jo Vandesompele (jo.vandesompele@ugent.be)

Materials availability

This study did not generate new unique reagents.

Data and code availability

The raw RNA-sequencing data of the Human Biofluid RNA Atlas Project (Hulstaert et al, 2020) that was generated with the protocols explained here, have been deposited at the European Genome-phenome Archive (EGA) under accession number EGAS00001003917. All spike-normalized sequencing data can be readily explored in the interactive web-based application R2: Genomics analysis and visualization platform (http://r2.amc.nl), and via a dedicated accessible portal (http://r2platform.com/HumanBiofluidRNAAtlas). A flowchart of the analytical procedures used to preprocess RNA sequencing data generated with the mRNA captures library preparation technique can be found at https://github.com/OncoRNALab/RNAexome.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100475.

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AUTHOR CONTRIBUTIONS

N.N., P.M., J.V., G.S., S.K., and S.G. conceived the protocol. N.N. and K.V. performed the experiments. A.D. and E.H. optimized the plasma preparation protocol. C.E. designed probes against the spike-in controls. C.E. and A.M. analyzed experiments and designed the bio-informatics pipeline. E.H. and A.D. wrote the manuscript with contributions from all authors. P.M. and J.V. supervised the study and acquired funding. All authors approved the final version of the protocol.

DECLARATION OF INTERESTS

N.N. is an employee, P.M. a consultant, and J.V. a co-founder of Biogazelle, a clinical CRO providing human biofluid extracellular RNA sequencing. G.S., S.G., and S.K. are employees of Illumina, providing the TruSeq RNA Exome Kit.

REFERENCES

Decock, A., Anckaert, J., Everaert, C., Nuytens, J., Kuersten, S., Hendrix, A., Vandesompele, J., Mestdagh, P., Dhondt, B., Van Paemel, R., et al. (2020). Abstract B49: substantial performance differences among RNA purification kits and blood collection tubes in the extracellular RNA quality control study—important considerations for liquid biopsies. In Poster Presentations - Proffered Abstracts (American Association for Cancer Research), p. B49.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21.

Grossman, R., Abel, B., Angiuoli, S., Barrett, J., Bassett, D., Bramlett, K., Blumenthal, G., Carlsson, A., Cortese, R., DiGiovanna, J., et al. (2017). Collaborating to compete: blood profiling atlas in cancer (BloodPAC) consortium. Clin. Pharmacol. Ther. *101*, 589–592.

Hardwick, S.A., Chen, W.Y., Wong, T., Deveson, I.W., Blackburn, J., Andersen, S.B., Nielsen, L.K., Mattick, J.S., and Mercer, T.R. (2016). Spliced synthetic genes as internal controls in RNA sequencing experiments. Nat. Methods *13*, 792–798.

Hulstaert, E., Morlion, A., Avila Cobos, F., Verniers, K., Nuytens, J., Vanden Eynde, E., Yigit, N., Anckaert, J., Geerts, A., Hindryckx, P., et al. (2020). charting extracellular transcriptomes in the human biofluid RNA atlas. Cell Rep. *33*, 108552.

Kirschner, M.B., Kao, S.C., Edelman, J.J., Armstrong, N.J., Vallely, M.P., van Zandwijk, N., and Reid, G. (2011). Haemolysis during sample preparation alters microRNA content of plasma. PLoS One *6*, e24145.

Kirschner, M.B., Edelman, J.J.B., Kao, S.C.-H., Vallely, M.P., van Zandwijk, N., and Reid, G. (2013). The impact of hemolysis on cell-free microRNA biomarkers. Front. Genet. *4*, 94.

Markham, N.R., and Zuker, M. (2008). UNAFold: software for nucleic acid folding and hybridization. Methods Mol. Biol. *453*, 3–31.

Mestdagh, P., Hartmann, N., Baeriswyl, L., Andreasen, D., Bernard, N., Chen, C., Cheo, D., D'Andrade, P., DeMayo, M., Dennis, L., et al. (2014). Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat. Methods *11*, 809–815.

Shah, J.S., Soon, P.S., and Marsh, D.J. (2016). Comparison of methodologies to detect low levels of hemolysis in serum for accurate assessment of serum microRNAs. PLoS One *11*, e0153200.

Van Buren, T., Arwatz, G., and Smits, A.J. (2020). A simple method to monitor hemolysis in real time. Sci. Rep. *10*, 5101.

Van Peer, G., Mestdagh, P., and Vandesompele, J. (2012). Accurate RT-qPCR gene expression analysis on cell culture lysates. Sci. Rep. *2*, 222.

Verwilt, J., Trypsteen, W., Van Paemel, R., De Preter, K., Giraldez, M.D., Mestdagh, P., and Vandesompele, J. (2020). When DNA gets in the way: a cautionary note for DNA contamination in extracellular RNA-seq studies. Proc. Natl. Acad. Sci. U S A *117*, 18934–18936.