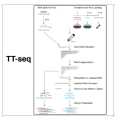

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Transient transcriptome sequencing: experimental protocol to monitor genome-wide RNA synthesis including enhancer transcription

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ABSTRACT

Transcriptome analysis by RNA sequencing (RNA-seq) measures the steady-state abundance of cellular RNA, which is a result of the interplay of RNA synthesis and RNA degradation. In order to measure RNA synthesis, RNA can be labeled with 4-thiouridine (4sU) in cells, purified and sequenced (4sU-seq). Although 4sU-seq has a higher sensitivity than RNA-seq, it is not sensitive enough to reliably detect short-lived (transient) RNAs such as enhancer, antisense, and promoter-associated RNAs synthesized from large genomes such as the human genome. This is because when the 4sU labeling time is less than 30 min only a short 3'-region of transcripts is labeled, and a long pre-existing unlabeled 5' region leads to a 5'-bias in the sequencing data. Transient transcriptome sequencing (TT-seq) overcomes this limitation by combining a short 4sU labeling pulse with an RNA fragmentation step. The labeled, newly synthesized RNA fragments are purified and sequenced, resulting in a very low fraction of contaminating non-labeled RNA. TT-seq is easy to use and includes RNA spike-in controls for global normalization between datasets from different samples. TT-seq enables studies of the kinetics of RNA metabolism, and mechanistic studies of transcription regulation. Also, TT-seq is ideally suited to monitor rapid changes in gene activity as well as the dynamics of enhancer landscapes during transcription responses.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

This manuscript will be published as a chapter in Methods in Molecular Biology.

MATERIALS

NAME	CATALOG #	VENDOR
MEGAscript® T7 Transcription Kit	AM1334	Thermo Scientific
KOD Hot Start DNA Polymerase	71086-3	Millipore Sigma
Qubit RNA HS Assay Kit	Q32852	Thermo Fisher Scientific
Qubit assay tubes	Q32856	Thermo Fisher Scientific
ERCC RNA Spike-In Mix or order custom-made synthetic sequences	4456740	Thermo Fisher Scientific
Transcriptor First Strand cDNA Synthesis Kit	04379012001	Roche
QIAquick PCR purification columns	28104	Qiagen
4-Thio-UTP	NU-1156S	Jena Bioscience
RNase-free AMPure XP beads	View	Beckman Coulter
4-thiouridine (4sU)	T4509	Sigma Aldrich
Cell scraper	3011	Corning
Chloroform	372978	Sigma Aldrich

NAME	CATALOG #	VENDOR
Ethanol	100983	Merck Millipore
Isopropanol	109634	Merck Millipore
1.5 mL Bioruptor Plus TPX micro tubes	C30010010-300	Diagenode
Screw Cap Micro Tube 2 mL polypropylene (PP)	72.694.005	Sarstedt
EZ-Link Biotin-HPDP	21341	Thermo Fisher Scientific
15 mL MaXtract High Density tubes	129065	Qiagen
µMACS Streptavidin Kit	130-074-101	Miltenyi Biotec
DL-dithiothreitol (DTT)	43815	Sigma Aldrich
miRNeasy Micro Kit	217084	Qiagen
RNase-Free DNase Set	79254	Qiagen
Sodium Acetate Solution (3 M) pH 5.2	R1181	Thermo Fisher Scientific
NaCl (5 M) RNase-free	AM9759	Thermo Fisher Scientific
Bioanalyzer RNA 6000 Nano Kit	5067	Agilent Technologies
Ovation Universal RNA-Seq System (NuGEN)	View	Tecan
AnyDeplete (formerly InDA-C)	View	Tecan
KAPA Library Quantification Kit	View	Kapa Biosystems
Bioanalyzer DNA 1000 Chip	5067	Agilent Technologies

MATERIALS TEXT

All materials, consumables and chemicals must be sterile, RNase-free, molecular biology grade.

It is essential to use RNase-free equipment.

Buffers and Stock Solutions

1 Prepare [M]**50 mM** **4-thiouridine (4sU) stock solution**. Dissolve 4sU in sterile RNase-free PBS. Store in small aliquots at **-20 °C** .

Thaw 4sU only once, and use immediately. As 4sU is photoactivatable and crosslinks to proteins at 365 nm, avoid light sources that emit this wavelength. The incorporation of 4sU can be validated by **dot blot** (see e.g. Radle et al. 2013).

2 Prepare **EZ-link HPDP-biotin stock solution**. EZ-link HPDP-biotin is pyridyldithiol-activated and allows thiol-specific biotinylation of labeled RNA. Dissolve in dimethylformamide (DMF) to [M]**1 mg/mL** , mix thoroughly and incubate at **37 °C** until it is completely dissolved. Repeat mixing if needed. Store in small aliquots in 2 mL PP micro tubes at **-20 °C**.

Please refer to safety data sheet for hazardous identification of DMF. Always wear gloves and eye protection. Avoid

contact with skin or clothing. Use in a chemical hood. Avoid breathing vapor. DMF should not get in contact with incompatible plastic materials. Eluted plastic might be carried along and causes substantial loss of labeled RNA.

Regarding the use of MTS-biotin (Duffy et al. 2015) in replacement of HPDP-biotin. We performed extensive control experiments to evaluate the described use of MTS-biotin in replacement of HPDP-biotin for our TT-seq protocol. For instance, we used 4sU-free total cellular RNA spiked-in with 0.1 % of 4sU labeled spike-ins, to assess the specificity of both biotins. When using MTS-biotin we found high levels of cross-contamination in the 4sU-pulldown fraction with unlabeled cellular RNA. This is in agreement with Marzi and Nicassio 2018 who detected 10-fold higher background levels for MTS-biotin compared to HPDP-biotin. Recently an improved MTS-biotin protocol including optimized biotin chemistry (MTS-resin) was developed (Duffy et al. 2018). As we have not yet tested this improved biotinylation protocol, we recommend to carry out careful control experiments before using this alternative for TT-seq.

3 Prepare **10x biotinylation buffer** containing [M]**100 mM** Tris HCl pH 7.4 and [M]**10 mM** EDTA pH 8.0. Store in small aliquots at **4 °C**.

4 Prepare **µMACS wash buffer** containing [M]**100 mM** Tris HCl pH 7.4, [M]**10 mM** EDTA pH 8.0, [M]**1 M** NaCl, [M]**0.1 %** (vol/vol) Tween20.

5 Prepare [M]**1 M** **DL-dithiothreitol (DTT)** in RNAse-free H_2O. Store in small aliquots at **-20 °C**. Thaw only once, and dilute to [M]**100 mM** before use.

6 Prepare miRNeasy Micro Kit **buffer RWT with isopropanol** instead of ethanol (see manufacturer's instructions for "DNase digestion for samples containing <1 µg total RNA approximately").

Spike-in Pool Preparation (based on Schwalb et al. 2016)

7 Six synthetic RNA spike-in controls (three unlabeled and three 4sU labeled spike-ins) were established for validation of labeled RNA enrichment, global normalization and for estimating possible cross-contamination from unlabeled RNA fragments. The spike-ins are derived from selected RNAs of the ERCC RNA Spike-in Mix (Schwalb et al. 2016). We chose six spike-ins of about 1,000 nt length, a GC content between 40 to 60 % and similar uridine content. The IVT simulates the *in vivo* labeling of the cells, assuming that one out of ten uridine bases is substituted with 4sU. If polyadenylated RNA spike-ins are required, add a poly(A) tailing step after PCR amplification, or perform the IVT directly from custom-made DNA sequences containing poly(A) tails.

Selected RNA spike-ins derived from the ERCC RNA Spike-in Mix.

ERCC ID	Length (nt)	%UTP	%4sUTP	%GC	Name (TT-seq)
00043	985	90	10	34	Spike-in 1
00170	949	100	0	35	Spike-in 2
00136	1,014	90	10	43	Spike-in 3
00145	1,015	100	0	46	Spike-in 4
00092	1,079	90	10	52	Spike-in 5
00002	1,037	100	0	53	Spike-in 6

Steps 8-12 can be omitted by ordering custom-made DNA sequences.

8 Perform first strand cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit with Anchored-oligo (dT) primers. Follow

manufacturer's instructions, use 1 µl ERCC RNA Spike-in Mix.

9 Amplify specific spike-ins from 1 µl of **1:4 diluted** cDNA using KOD Hot Start DNA Polymerase following manufacturer's instructions (30 cycles).

Primer sequences to amplify the cDNA template for IVT reaction of RNA spike-ins.

Primer name (TT-seq)	Sequence 5'>3'	Annealing temperature (°C)
Spike-in 1 forward	TAATACGACTCACTATAGGGTGCTTTAACAAGAGGAAATTGTGT	53
Spike-in 1 reverse	CCATCTTGTTTATAAAATCCTAATTACTC	53
Spike-in 2 forward	TAATACGACTCACTATAGGGGGCACAAGTTGCTGAAGTTGC	55
Spike-in 2 reverse	TCTGCTGTAATCTCAGCTCC	55
Spike-in 3 forward	TAATACGACTCACTATAGGGTTTCGACGTTTTGAAGGAGGG	53
Spike-in 3 reverse	GTACCCGGGAAAATCCTAGTTC	53
Spike-in 4 forward	TAATACGACTCACTATAGGGACTGTCCTTTCATCCATAAGCGG	55
Spike-in 4 reverse	CGCACGCCGAATGATGAAACG	55
Spike-in 5 forward	TAATACGACTCACTATAGGGGATGTCCTTGGACGGGGT	55
Spike-in 5 reverse	GCTTTCGGAGCAAATCGCG	55
Spike-in 6 forward	TAATACGACTCACTATAGGGCCAGATTACTTCCATTTCCGCC	55
Spike-in 6 reverse	GGGTAAAACGCAAGCACCG	55

10 Purify PCR products with QIAquick PCR purification columns following manufacturer's instructions. Elute in 30 µl.

11 Control size and purity of PCR products by gel electrophoresis.

Optional cDNA purification: if PCR products show contamination with smaller fragments, isolate the PCR product of the desired size by gel extraction (e.g. by QIAquick Gel Extraction Kit).

12 Verify sequence of PCR products by Sanger sequencing.

13 Use 0.5 µg PCR product (or, custom-made DNA) as input for *in vitro* transcription (IVT) using the MEGAscript T7 kit. Follow manufacturer's instructions, **except for IVT of labeled RNA spike-ins (referred to as 1, 3, 5) substitute** [M]10 % **of UTP with 4-Thio-UTP.**

14 Purify RNA spike-ins with RNase-free AMPure XP beads following manufacturer's instructions.

15 Use the Qubit HS RNA Kit and Agilent RNA 6000 Nano Kit for quantification and quality assessment.

16 For the final RNA spike-in pool containing equimolar amounts of all six RNA spike-ins, add each RNA spike-in to a final concentration of [M]1 ng/µL .

17 Assess the actual concentration of your RNA spike-in pool by Qubit HS RNA Kit and use this concentration as your working concentration.

18 Store RNA spike-in pool in small, one-time use aliquots at -80 °C (see **Step 25** for the required amount of spike-ins per sample).

Cell Treatment and 4sU Labeling

19 The required number of cells depends on the cell line under study. The exact cell count is required to calculate the corresponding amount of RNA spike-ins.

Depending on cell type specific parameters (doubling time, transcriptional activity, and amount of cellular RNA), between 300 to 600 µg total RNA are needed to isolate sufficient amounts of labeled RNA (> 50 ng). To establish a 4sU-labeling experiment, determine first how many cells are needed to yield 300 (or 600 µg) of total RNA and then, how much labeled RNA can be isolate from 300 µg (or 600 µg) input RNA. Based on these results, calculate the required number of cells. Typically, for 5 min of 4sU labeling the required number of cells is 1.5 to 5 x 10^7. At the time of labeling cells should still be in their exponential growth phase to ensure high transcriptional activity. Therefore, we label cells at around 70 % confluence.

20 Optional: add treatment prior to 4sU labeling.

Labeling of control and treated cells: when comparing TT-seq of control and manipulated cells it is critical that cell densities and labeling times are kept consistent for comparable 4sU uptake and incorporation.

21 **Labeling of Adherent Cells**

21.1 24-48 h before the experiment, plate the required number of cells in 15 cm dishes (20 ml culture medium). Seed 1-2 additional plates for cell counting.

21.2 00:30:00 before labeling thaw 4sU stock solution on ice and count cells.

21.3 For 4sU labeling, incubate cells with a final concentration of [M]500 µM 4sU at 37 °C, [M]5 % CO_2 for exactly 00:05:00 .

4sU labeling of adherent cells: just before labeling, pipet the required amount of 4sU for each plate into a sterile 50 mL tube. Remove half of the culture medium from each plate (it is important to leave some volume behind to not stress the cells) and add to the 50 mL 4sU-tube, mix and immediately pour back. Do not handle more than three plates in parallel.

4sU concentration and labeling time can be adjusted to yield more labeled RNA. However, it is important to balance labeling concentration and time to avoid possible inhibition of rRNA synthesis (Burger et al. 2013) and cell toxicity.

21.4 After 00:05:00 quickly remove culture medium and add 4.5 ml of TRIzol lysis reagent.

Please refer to safety data sheet for hazardous identification of TRIzol lysis reagent. Always wear gloves and eye protection. Avoid contact with skin or clothing. Use in a chemical hood. Avoid breathing vapor.

21.5 "Wash" cells off the plate by pipetting up and down. If cells are very adherent, use a TRIzol compatible cell scraper.

21.6 Transfer cell lysate to 15 mL polypropylene (PP) centrifuge tube and incubate up to 00:05:00 .

Use centrifuge tubes for high speed (≥13,000 g) centrifugation.

If several plates are labeled simultaneously, two lysates from the same condition can be pooled into one PP tube (minimizes the number of tubes).

21.7 Optional: store lysates at -80 °C.

22 Labeling of Suspension Cells

22.1 Dilute cells 48 h before the labeling experiment and exchange growth medium one day before labeling.

22.2 00:30:00 before labeling thaw 4sU stock solution on ice and count cells.

22.3 Add 500 µl of 4sU stock solution to cells in 50 ml of growth medium to a final concentration of [M]500 µM , and incubate at 37 °C, [M]5 % CO_2.

22.4 Exactly after 00:05:00 of labeling, centrifuge at 37 °C and 500-1,500 x g for 00:02:00 . Discard supernatant by decanting.

22.5 Add **5 ml** of TRIzol lysis reagent to cell pellet. Vortex until no cells are visible (**00:00:30**).

22.6 Incubate for **00:05:00** . Transfer to 15 mL PP centrifuge tubes.

Use centrifuge tubes for high speed (≥13,000 x g) centrifugation.

22.7 Optional: store lysates at **-80 °C**.

RNA Spike-in Pool Addition and Total RNA Extraction

23 If lysates were frozen, thaw quickly at **65 °C** (water bath), then cool on ice for **00:05:00** .

24 Thaw RNA Spike-in mix at **65 °C** for **00:02:00** , cool on ice for **00:01:00** and mix gently by pipetting.

25 Add **2.4 ng** spike-in mix per 10^6 cells to TRIzol cell lysate.

RNA spike-in addition to cell lysate: the amount of RNA spike-ins depends on the complexity and concentration of the final sample, but also on how deep the library is sequenced. In general, a balance between detecting the spike-ins and not having too many sequencing reads for spike-ins is recommended.

26 Vortex gently and incubate for **00:05:00** .

27 Add **0.2 ml** of chloroform **per** **1 ml** of TRIzol lysis reagent.

28 Shake vigorously for **00:00:15** and incubate for **00:03:00** .

29 Centrifuge at **4 °C** and **13000 x g** for **00:15:00** .

30 Carefully transfer upper, aqueous phase into new 15 mL centrifuge tube.

31 Add an **equal volume** of isopropanol to precipitate RNA, vortex gently.

32 Incubate for **00:10:00** .

33 Centrifuged at 4 °C and 13000 x g for 00:10:00 .

34 Wash RNA pellet twice, first using an **equal volume**, then 1 ml of [M]75 % ethanol. Spin in-between at 4 °C and 13000 x g for 00:10:00 .

35 Remove supernatant with 1 mL pipette. Spin down briefly and use 200 µL pipette to remove remaining ethanol. Eventually, spin down again and use 10 µL pipette.

36 Dissolve pellet in **200-1,000 µL** H_2O (aim for a final concentration of ≥ 750 ng/µL).

37 Transfer RNA to 1.5 mL tube.

Optional: pool samples from the same condition into one tube.

38 Determine concentration and purity by NanoDrop spectrophotometer; 260/280 value should be >2.

39 **Save** 1 µl of total RNA for quality control on Agilent Bioanalyzer RNA 6000 Nano Chip (**Step 45**).

40 Optional: store at -80 °C.

Mild RNA Fragmentation (based on Schwalb et al. 2016)

41 Cool Bioruptor Plus to 4 °C before use. Transfer 300 µg of total RNA to 1.5 mL Bioruptor Plus TPX microtubes, adjust to [M]750 ng/µL .

If processing 600 µg RNA: use two 1.5 mL Bioruptor Plus TPX microtubes (300 µg each).

RNA fragmentation using Covaris S220: transfer two-times 150 µg of total RNA (each in a final volume of 130 µl) to 130 µL microTUBE AFA Fiber Snap-Cap tubes. Use the following settings: water level 12, temperature 7 °C, peak incident power 100 W, duty factor 1 %, cycles per burst 200, treatment time 10-60 sec.

42 Fill empty positions in the tube holder with tubes containing 400 µl water.

43 Settings: 1 cycle, 30 sec ON, 30 sec OFF at HIGH power.

44 Transfer (optional: pool) samples to 2 mL PP microtubes. Continue immediately with the biotinylation reaction.

45 After fragmentation, use 1 µl of total fragmented RNA for quality control on Agilent Bioanalyzer RNA 6000 Nano Chip and compare to 1 µl of total RNA. For expected results see **Step 94**.

Biotinylation of 4sU Labeled RNA (based on Dölken et al. 2008)

46 Denature total fragmented RNA at 65 °C for 00:10:00, then place on ice for 00:05:00.

47 **Save** 5 µl of total fragmented RNA for DNase I digest (**Step 75**).

48 Split remaining total fragmented RNA in **two** 2 mL PP microtubes (150 µg/reaction) and process in parallel in **Steps 49-53**.

If processing 600 µg RNA: split total fragmented RNA in **four** 2 mL PP microtubes (150 µg/reaction) and process in parallel (**Steps 49-53**).

49 Add water **up to** 700 µl.

50 Prepare **biotin master mix** in the stated order: 100 µl of 10x biotinylation buffer, 200 µl of EZ-link HPDP-Biotin stock solution.

51 Add 300 µl biotin master mix to each sample and mix immediately.

52 Incubate in the dark with rotation for 02:00:00.

53 Shortly before use, prepare 15 mL MaXtract High Density tubes following the manufacturer's instruction.

54 Transfer biotin-RNA mix to MaXtract High Density tubes, **pool reactions from the same condition**.

55 Add an **equal volume** of chloroform. Mix vigorously for 00:00:15 and incubate for 00:03:00.

56 Centrifuge at 1500 x g for 00:05:00.

57 Transfer upper phase into 15 mL PP centrifuge tube.

58 For RNA precipitation, add **1/10 volume** of [M]5 M NaCl and an **equal volume** of isopropanol. Vortex gently.

59 Centrifuge at 4 °C and 13000 x g for 00:30:00 . Remove supernatant.

60 Wash RNA pellet twice, first using an **equal volume**, then 1 ml of [M]75 % ethanol. Spin in-between at 4 °C and 13000 x g for 00:10:00 .

61 Remove supernatant with 1 mL pipette. Spin down briefly and use 200 µL pipette to remove remaining ethanol. Optionally, spin down again and use 10 µL pipette.

62 Resuspend RNA in 100 µl H_2O per 300 µg biotinylation reaction: chill on ice 00:10:00 , then heat at 65 °C for 00:10:00 , put on ice and carefully resuspend by pipetting. Transfer to 2 mL LoBind tubes.

63 Continue immediately with 4sU pull-down.

Pull-down of 4sU Labeled RNA Using Streptavidin-Beads

64 Use one µMACS column per 300 µg (200 µl) RNA.

If processing 600 µg RNA: use **two** µMAC columns with 300 µg (200 µl) RNA each.

65 Heat µMACS wash buffer (3 ml/µMAC column) to 65 °C.

66 Add 100 µl of µMACS streptavidin beads to 200 µl RNA.

67 Incubate at 4 °C with rotation for 00:15:00 .

68 In the meantime, equilibrate µMACS columns with 100 µl of nucleic acids equilibration buffer (equilibrate at RT 00:10:00 before use).

69 Transfer RNA-bead-mix to the µMACS column, **collect the flow-through** in LoBind tubes and reapply to the µMACS column. Discard flow-through.

70 Wash 3-times with 900 µl of **µMACS wash buffer** (65 °C). Discard flow-through.

71 Wash 3-times with 900 µl of **µMACS wash buffer** (Room temperature). Discard flow-through.

72 Place 1.5 ml LoBind tubes underneath the columns.

73 Elute labeled RNA in 100 µl of [M]100 mM DTT.

74 After 00:05:00 elute in additional 100 µl of [M]100 mM DTT.

Final RNA Clean-up and DNase Digest

75 Prepare 1 µg total fragmented RNA (**Step 47**). Mix samples thoroughly by shaking for 00:00:15 .

Total fragmented RNA (1 µg)	200 µL
3 M NaAc pH 5.2	15 µL
100 % ethanol	300 µL

From here on use low binding pipette tips. Process labeled RNA and 1 µg of total fragmented RNA in parallel.

76 Prepare labeled RNA (**Step 74**). Mix samples thoroughly by shaking for 00:00:15 .

4sU labeled RNA	200 µL
100 % ethanol	300 µL

77 Transfer samples onto miRNeasy MinElute spin column in a 2 mL collection tube.

If processing labeled RNA isolated from 600 µg total fragmented RNA: pool samples from the same condition onto **one** RNeasy MinElute spin column by repeating **Step 78**.

78 Centrifuge at 8000 x g for 00:00:15 . Reload flow-through.

79 Wash with 350 µl of RWT buffer by centrifugation at 8000 x g for 00:00:30 .

80 Prepare DNase mix: 70 µl RDD buffer and 10 µl RNase-free DNase I, mix by pipetting or inverting.

81 Add 80 µl DNase master mix to each column and incubate for 00:15:00 .

82 Add 500 µl of RWT buffer.

83 Centrifuge at 8000 x g for 00:00:30 . **Reload flow-through.**

84 Centrifuge at 8000 x g for 00:00:30 . Discard flow-through.

85 Add 500 µl of RPE buffer.

86 Centrifuge at 8000 x g for 00:00:30 .

87 Place column into a new 2 mL collection tube.

88 Wash with 500 µl of [M]80 % ethanol. Centrifuge at 8000 x g for 00:02:00 . Place column into new 2 mL collection tube.

89 To dry the membrane, open the lid and centrifuge at full speed for 00:05:00 .

90 Place column into new 1.5 mL LoBind tube.

91 Elute with 15 µl of nuclease free H_2O (not DEPC treated) by centrifugation at full speed for 00:01:00 . Reload flow-through for a second elution. If a low labeled RNA yield is expected: use 11 µl of H_2O and incubate 00:01:00 before centrifugation.

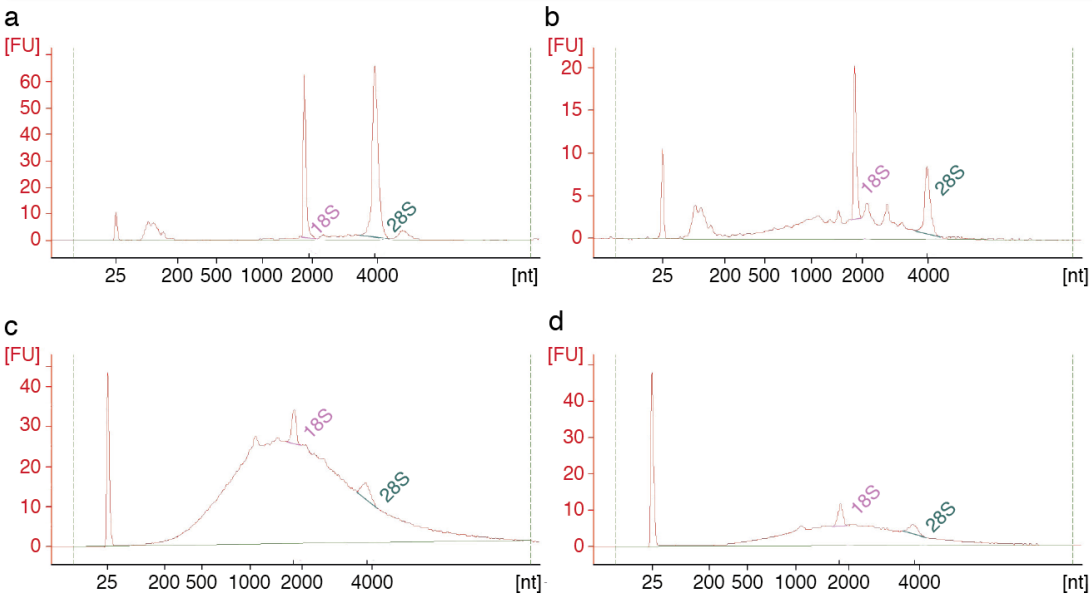
92 Transfer RNA to a new 1.5 mL LoBind tube.

93 Measure RNA concentration with Qubit RNA HS Kit and analyze RNA integrity on Agilent Bioanalyzer RNA 6000 Pico Chip.

Expected Result

94 **Examples of RNA integrity profiles of total, total fragmented and labeled RNA isolated from human cell lines.**

Total fragmented RNA should be in a range between 0.2 and 15 kbp. Fragmentation efficiency might differ for RNA from another organisms. Thus, it is recommended to perform initial trials with your cell lines. Be aware that after sonication it is expected that the RIN value drops since rRNA is also fragmented.



Agilent Bioanalyzer profiles of total RNA (a) and total fragmented RNA (b) on a RNA 6000 Nano Chip. Agilent Bioanalyzer profiles of labeled RNA on a RNA 6000 Pico Chip from a cell line with high (c) and low (d) amounts of labeled RNA.

95 **Highly recommended: validation of labeled RNA enrichment by reverse transcription PCR (RT-qPCR) of RNA spike-ins.**

Control of labeled RNA enrichment by RT-qPCR. Prepare cDNA from **400 ng** total fragmented RNA and **1 µl** labeled RNA, and perform RT-qPCR. Use primer pairs for RNA spike-ins to estimate labeled RNA enrichment, and (optional) for endogenous model genes of your choice to measure the intron enrichment.

Primer sequences for RT-qPCR of RNA spike-ins to control enrichment of labeled RNA.

Primer name (TT-seq)	Sequence 5'>3'	Annealing temperature	Amplicon size (bp)
Spike-in 1 forward	ACAATTCCAAATAGCGACCA CATCA	59	150
Spike-in 1 reverse	TACCTCAACCCTTCCAGTGT CTAAG	58	
Spike-in 2 forward	AGACTGGCATTCCCGTGATA	57	97
Spike-in 2 reverse	GCTAAAACCCCTGCCTGCAA	60	
Spike-in 3 forward	CCGAGTTCGCCTTACTGCTC	60	95
Spike-in 3 reverse	AATCGATCGGAATCACGCCG	60	
Spike-in 4 forward	CATAAGCGGAGAAAGAGGG AATGAC	59	103

Spike-in 4 reverse	GCTAAATAGAGAGCATCCACACCTC	58	
Spike-in 5 forward	CGTTAATGCAGAGGCTAAGGACAAT	59	103
Spike-in 5 reverse	GATCGTTACAAACCCACTACGTGTC	59	
Spike-in 6 forward	GTCCTGATTTACTGGACTCGCAAC	58	118
Spike-in 6 reverse	TCTGTATAAGGTGATCGCAGGTTGT	59	

For total fragmented RNA, the Ct values of labeled and unlabeled RNA spike-ins are very similar. Labeled RNA shows a Ct value difference of 6-10 for labeled and unlabeled RNA spike-ins. For the selected model gene, intron levels should be enriched in labeled RNA compared to total fragmented RNA.

Library Preparation

96 We recommend using **100 ng** (at least **50 ng**) labeled RNA and **100 ng** total fragmented RNA as library input.

We recommend using the Ovation Universal RNA-Seq System (NuGEN). The option of using only random primers for cDNA synthesis omits the 3' bias generated by poly-(dT) primers. Also, the NuGEN kit depletes ribosomal RNA by sequence-specific probes (AnyDeplete technology, formerly known as InDA-C).

Total fragmented RNA samples are needed to estimate RNA half-lives and correct the cross-contamination using RNA spike-ins. If the cross-contamination rate is similar across your libraries (which can be estimated from the RNA spike-ins), labeled RNA might be sufficient.

97 For 'First Strand Primer Pre-mix Preparation', use only Random primer mix. Substitute poly-d(T) primers with **0.4 µl** H_2O.

98 Follow the User guide's instruction from 'First Strand Synthesis Using DNase-treated RNA' to 'Second Strand cDNA Synthesis'.

99 For 'cDNA fragmentation' (200-400 bp) using a Bioruptor Plus device: Aliquot **100 µl** cDNA in Qubit assay tubes. Fill up empty positions in the tube holder with tubes containing **100 µl** water. Do 15 cycles, 30 sec ON, 30 sec OFF at LOW power. Spin down samples and add another 10 cycles. If the Bioruptor is not connected to an automated cooling system. Cool the water bath with ice in-between the 15 and 10 cycles.

cDNA fragmentation using Covaris S220 (200-400 bp): transfer cDNA to 130 µL microTUBE AFA Fiber Snap-Cap tube. Use the following settings: water level 12, temperature **7 °C**, peak incident power 145 W, duty factor 10 %, cycles per burst 200, treatment time 50-180 sec.

100 Follow the User guide's instruction from 'cDNA Concentration After Fragmentation' to 'Adaptor Cleavage'.

101 Before 'PCR amplification': To avoid over-amplification determine the precise number of PCR cycles by the KAPA HIFI Library Amp Real Time kit following manufacturer's instructions. As input, use **4.2 µl** (~10 %) of the library, **5 µl** of 2x Kapa HiFi Hot Start Master Mix and **0.8 µl** Amplification Primer Mix P2 (NuGEN).

102 For 'PCR amplification' of the remaining **45 µl** of library use the determined number of PCR cycles and follow the User guide's instructions for amplification as described in the Appendix 'Using qPCR to determine the Number of PCR Cycles'.

103 Proceed from 'Bead Purification of the Amplified Material' to 'Quantitative and Qualitative Assessment of the Library'.

104 Assess the quality of the library on Bioanalyzer DNA 1000 Chip and send to sequencing service providers for high throughput sequencing.

Paired-end Illumina Sequencing

105 Universal RNA-Seq with NuQuantThe read length for human transcriptomes should be between 35 to 150 bp depending on the desired application. For alternative splicing detection, longer reads are recommended, i.e. 150 bp paired-end sequencing. Recommended coverage: in general, 25 to 50 million reads per sample are sufficient for an initial analysis of highly expressed transcripts. 100 million reads is the required sequencing depth for annotation of enhancer RNAs or antisense RNAs in TT-seq (labeled RNA) samples (see Deveson et al. 2017 for review). If the focus of a study is on newly synthesized RNA fragments, total fragmented RNA-seq libraries can be sequenced with a lower coverage, i.e. 30 million reads. If a study aims to study RNA stability, TT-seq and total fragmented RNA-seq libraries should be sequenced at similar coverage.

Computational analysis

106 The bioinformatics workflow used to analyze TT-seq data is presented in Villamil et al. 2019. The pre-print describe pre-processing steps, including a reliable and robust normalization strategy, and several downstream analysis tools that enable the user to quantify RNA synthesis, splicing and degradation activities.