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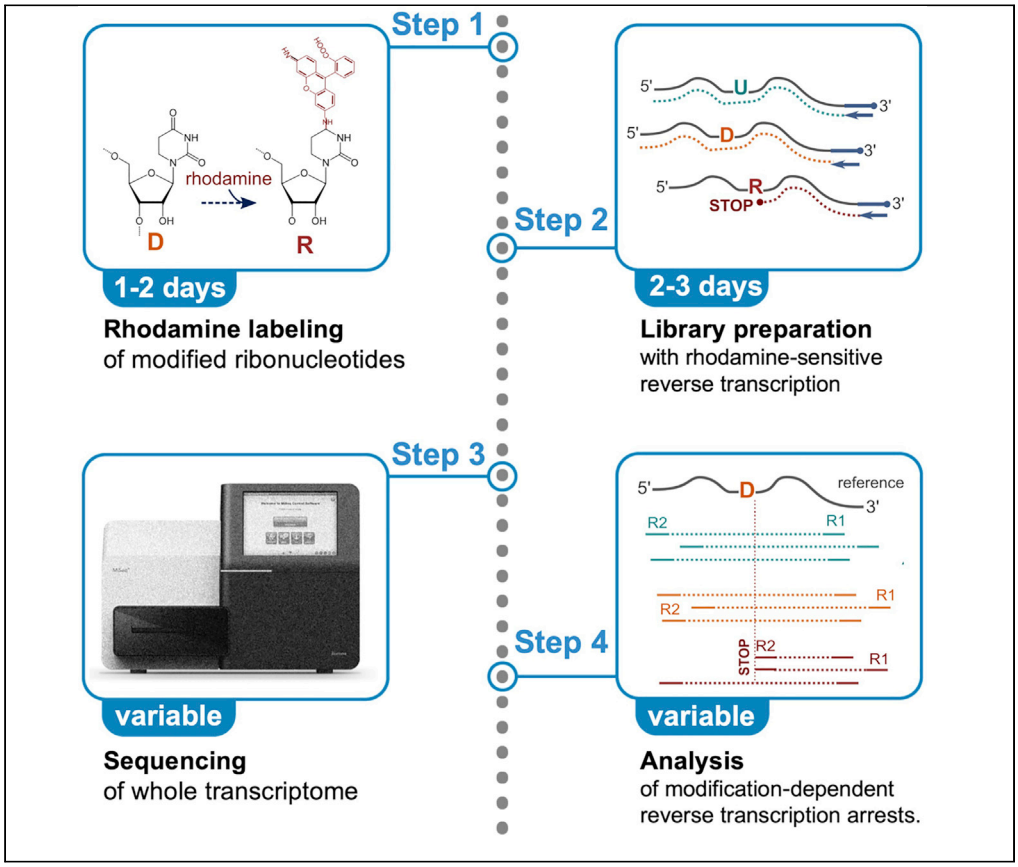
Epitranscriptomic mapping of RNA modifications at single-nucleotide resolution using rhodamine sequencing (Rho-seq)



The recent development of epitranscriptomics revealed a new fundamental layer of gene expression, but the mapping of most RNA modifications remains technically challenging. Here, we describe our protocol for Rho-Seq, which enables the mapping of dihydrouridine RNA modification at single-nucleotide resolution. Rho-Seq relies on specific rhodamine-labeling of a subset of modified nucleotides that hinders reverse transcription. Although Rho-Seq was initially applied to the detection of dihydrouridine, we show here that it is applicable to other modifications including 7-methylguanosine or 4-thiouridine.

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Highlights

Rho-seq maps dihydrouridine (D) RNA modification at a 1-nucleotide resolution

Rho-seq relies on labeling D with rhodamine that blocks reverse transcription

We describe how Rho-seq can be adapted to study m^7G, S^4U or $cmnm^5S^2U$

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Epitranscriptomic mapping of RNA modifications at single-nucleotide resolution using rhodamine sequencing (Rho-seq)

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SUMMARY

The recent development of epitranscriptomics revealed a new fundamental layer of gene expression, but the mapping of most RNA modifications remains technically challenging. Here, we describe our protocol for Rho-Seq, which enables the mapping of dihydrouridine RNA modification at single-nucleotide resolution. Rho-Seq relies on specific rhodamine-labeling of a subset of modified nucleotides that hinders reverse transcription. Although Rho-Seq was initially applied to the detection of dihydrouridine, we show here that it is applicable to other modifications including 7-methylguanosine or 4-thiouridine.

For complete details on the use and execution of this protocol, please refer to Finet et al. (2022).

BEFORE YOU BEGIN

General experimental considerations

Until recently, the dihydrouridine (D) RNA modification was exclusively studied within the context of tRNA and rRNA biology. However, preliminary data from our lab and elsewhere led us to consider the possibility that other RNA species could also be dihydrouridilated (Dudin et al., 2017; Fauquenoy et al., 2018; Finet et al., 2022). To assess whether D extends beyond tRNA is particularly challenging given the close resemblance of D to the canonical nucleotide U. However, pioneer work reported a chemical reaction specifically acting on D allowing its labeling with the rhodamine fluorophore (Betteridge et al., 2007). We reasoned that regardless of the fluorescent properties of rhodamine, its incorporation should interfere with reverse transcription (RT), leaving a footprint that can be exploited to indirectly observe the presence D. In earlier epitranscriptomic studies, the concept of RT stop footprint was successfully applied to the detection of pseudouridine (Schwartz et al., 2014), inosine (Suzuki et al., 2015) and m^1A (Li et al., 2017; Safra et al., 2017). Similarly, by combining rhodamine labeling of the D modification with high-throughput sequencing, we developed Rho-seq, a method to assess the presence of D at the transcriptome-wide scale (Finet et al., 2022).

Rho-seq relies on the specific reduction of D with sodium borohydride ($NaBH_4$) (Cerutti and Miller, 1967) followed by the incorporation of a bulky rhodamine molecule that arrests RT one nucleotide downstream of the modified position (Kaur et al., 2011). By comparing patterns of RT stops between $NaBH_4$-treated (R^+) and mock-treated samples (R^-), Rho-seq allows the detection of putative D-sites. However, while most major nucleotides are inert toward $NaBH_4$ (Cerutti and Miller, 1967), other modifications react to it, including N4-acetylcytidine (ac^4C),

Table 1. Application of rho-seq to multiple RNA modifications

Modification	Reacts with NaBH4	NH_2-dye incor-poration	Comment	Genes involved in the RNA Modification metabolism			References
				E. coli	*S. pombe*	*H. sapiens*	
m^1A	✓	?	–	–	Gcd10, Gcd14, Bmt2, Rrp8	TRMT6, TRMT16A|B, TRMT10C, BMT2, RRP8	(Cerutti et al., 1968; Macon and Wolfenden, 1968; Igo-Kemenes and Zachau, 1969)
m^3C	✓	?	–	–	Trm140, Trm141	METTL2, METTL6, METTL8	(Cerutti et al., 1968; Macon and Wolfenden, 1968)
f^6A	✓	?	–	–	–	FTO	(Cerutti and Miller, 1967)
ac^4C	✓	?	–	TmcA	Nat10	NAT10	(Cerutti and Miller, 1967; Cerutti et al., 1968; Molinaro et al., 1968; Igo-Kemenes and Zachau, 1969)
yW	✓	✓	yW stops RT (R-independent) in Rho-seq	–	Tyw1, Trm12, Tyw3, Ppm2	TYW1, TRMT12, TYW3, LCMT2	(Igo-Kemenes and Zachau, 1969; Beltchev and Grunberg-Manago, 1970; Wintermeyer and Zachau, 1974; Schleich et al., 1978)
m^7G	✓	✓	R^+-dependent RT stops in Rho-seq (Figure 2A)	RlmL, RsmG, TrmN	Pcm1, Bud23, Trm8, Trm82	RNMT, BUD23, METTL1, WDR4	(Igo-Kemenes and Zachau, 1969; Wintermeyer and Zachau, 1974)
s^4U	✓	?	R^+-dependent RT stops in Rho-seq (Figure 2C)	ThiI	–	–	(Cerutti and Miller, 1967; Igo-Kemenes and Zachau, 1969; Wintermeyer and Zachau, 1974)
$cmnm^5s^2U$	?	?	R^+-dependent RT stops in Rho-seq (Figure 2D)	MnmA	Slm3	TrmU	–
D	✓	✓	R^+-dependent RT stops in Rho-seq	DusA, DusB, DusC	Dus1, Dus2, Dus3, Dus4	Dus1, Dus2, Dus3, Dus4	(Cerutti and Miller, 1967; Betteridge et al., 2007)

For all modifications known to react with $NaBH_4$, it is indicated whether the incorporation of NH_2-dye (such as rhodamine) was previously reported and the appropriate references are indicated. The known or predicted RNA modifying enzymes in *E. coli*, *S. pombe* and *H. sapiens* are specified.

N6-formyladenosine (f^6A), 1-methyladenosine (m^1A), 3-methylcytidine (m^3C), 7-methylguanosine (m^7G), 4-thiouridine (S^4U), and wybutosine (yW) (Table 1). Therefore, to control against other $NaBH_4$-sensitive modifications, dihydrouridine-free RNA extracts obtained from a strain deleted for its D-synthases (*Δ4dus*) are also treated R^+ and R^- and compared to the wild-type (Figure 1).

Highlighting the importance of the *Δ4dus* control, we observed in our original *S. pombe* Rho-seq data the existence of *R^+-dependent* but *dus-independent* RT stop sites corresponding to the conserved 18S rRNA m^7G modification (Figure 2A). Furthermore, in *E. coli* Rho-seq data, we observed that *R^+-dependent* but *dus-independent* RT stop sites are preferentially located at the tRNAs positions 8 and 34 (Figure 2B). The position 8 on tRNAs is a S^4U modification site conserved in prokaryotes (Figure 2C), while the position 34 on tRNA Lys_{UUU} and tRNA Gln_{UUG} are $cmnm^5s^2U$ modification site (Boccaletto et al., 2018) (Figure 2D). Together, these analyses suggest that Rho-seq can be adapted to detect the presence of these other $NaBH_4$-sensitive modifications using appropriate modification-free controls (Table 1).

The protocol below describes the specific steps for using Rho-seq to detect dihydrouridine in fission yeast. However, we have also used Rho-seq in prokaryote (*E. coli*) and higher eukaryote (human cell line HCT116) (Finet et al., 2022) and the protocol can be readily applied to any organism provided that the appropriate modification-free controls are available (Table 1).

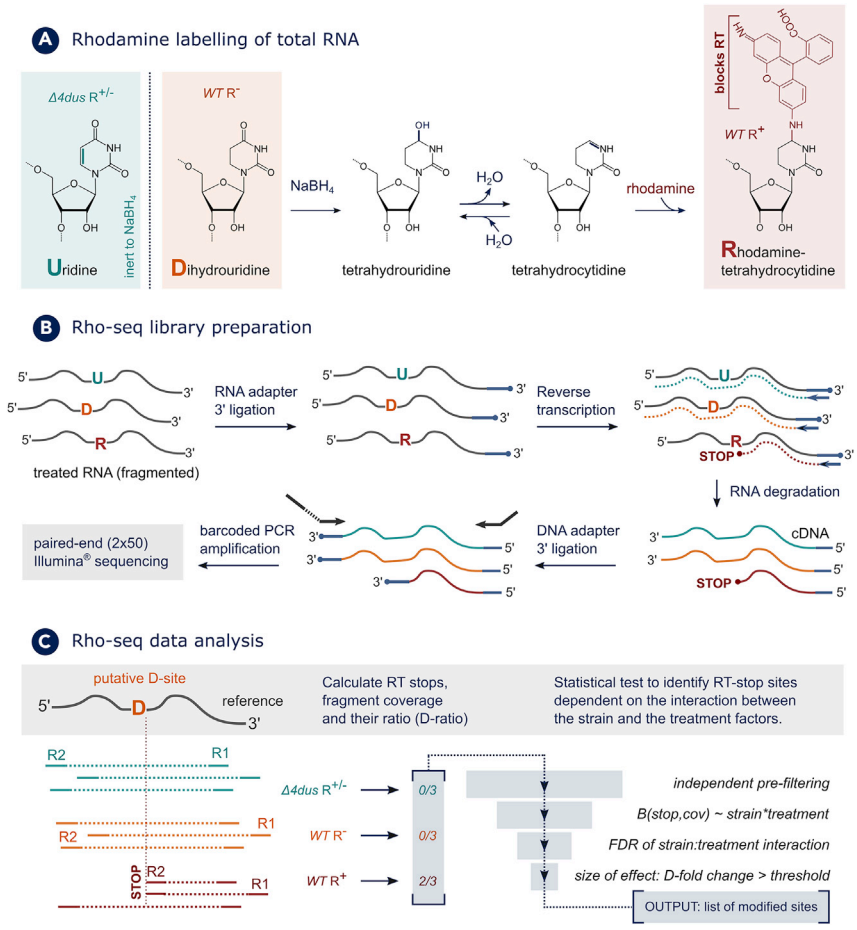

Figure 1. Overview of Rho-seq workflow

(A) A chemical treatment is applied to total RNA extract to label dihydrouridine (D) with rhodamine. It begins with the specific reduction of D with sodium borohydride ($NaBH_4$). Upon the addition of a nucleophile NH_2-fluorophore such as rhodamine in acidic condition, a covalent bond is formed with Schiff base intermediate tetrahydrocitidine (Kaur et al., 2011). Along with the effective rhodamine labeling condition (*WT* R^+, highlighted in red), control condition include mock labeling (*WT* R^-) where KOH substitute for $NaBH_4$ and D is therefore not reduced (R^-, highlighted in orange), as well as dihydrouridine-free total RNA extracts obtained from a strain where each dihydrouridine synthase is deleted (*Δ4dus* R^+ and R^- , highlighted in cyan blue). Importantly, other RNA modifications can be specifically labeled using the same strategy, including m^7G and s^4U.

(B) R^+ and R^- treated RNAs containing uridine only (U, in cyan blue) from the *Δ4dus* condition, dihydrouridine (D, in orange) from the WT R^- condition and rhodamine-tetrahydrocitidine (R, in red) from the WT R^+ condition are then subject to library preparation for high throughput sequencing. After RNA fragmentation, an RNA adapter blocked in 3′ with dideoxycytidine (blue line ending with a filled circle in 3′) is ligated to the fragmented RNA. Then, DNA primers complementary to the RNA adapter prime reverse transcription reactions. Reverse transcription either ends at the end of the RNA fragments (dotted lines in cyan and orange) or can be stopped prematurely by the bulky rhodamine molecule in the WT R^+ condition (dotted red line ending with a filled circle in 3′). Finally, after the ligation of a DNA adaptor (in cyan, also blocked in 3′) in 3′ of the cDNA, the library is amplified by PCR with primers complementary to the adapters flanked with additional barcoded sequences. The amplified library is finally sequenced according to Illumina® chemistry in paired-end.

(C) After trimming and mapping the sequenced paired-end reads to a reference sequence (R1 and R2 refer to the left and right read of a pair), the D-ratio is calculated at a single nucleotide resolution for each condition. It is the ratio between the number of reverse transcription stop events – reflected by the number of R2 reads starting at a position – and the fragment coverage. To robustly identify putative D-sites, i.e., transcriptomic position where the D-ratio is significantly higher in the WT R^+ condition while controlling for all other factors, a three-stage approach is applied. First, the sites are filtered based on unsupervised criterion independent of the test statistic (see main text for details). Then, the D-ratio of the remaining sites is modelized in a generalized linear model of the binomial family with a logit link where the treatment (R^+ and R^-), the strain (*WT* and *Δ4dus*) and their interaction are explanatory variables. The

Figure 1. *Continued*

p-value of the coefficient of the strain:treatment interaction is computed and corrected using the Benjamini-Hochberg procedure (FDR). Finally, the effect size (D-fold change) is calculated as the ratio between the average D-ratio of the WT R^+ condition against the average D-ratio of all the other control conditions.

Preparation of total RNA

Timing: 1 day

A minimum of 60 μg of total RNA extracted from both WT and modification-free mutant strains is required to perform rhodamine labeling (steps 1–10). The RNA should be of excellent quality

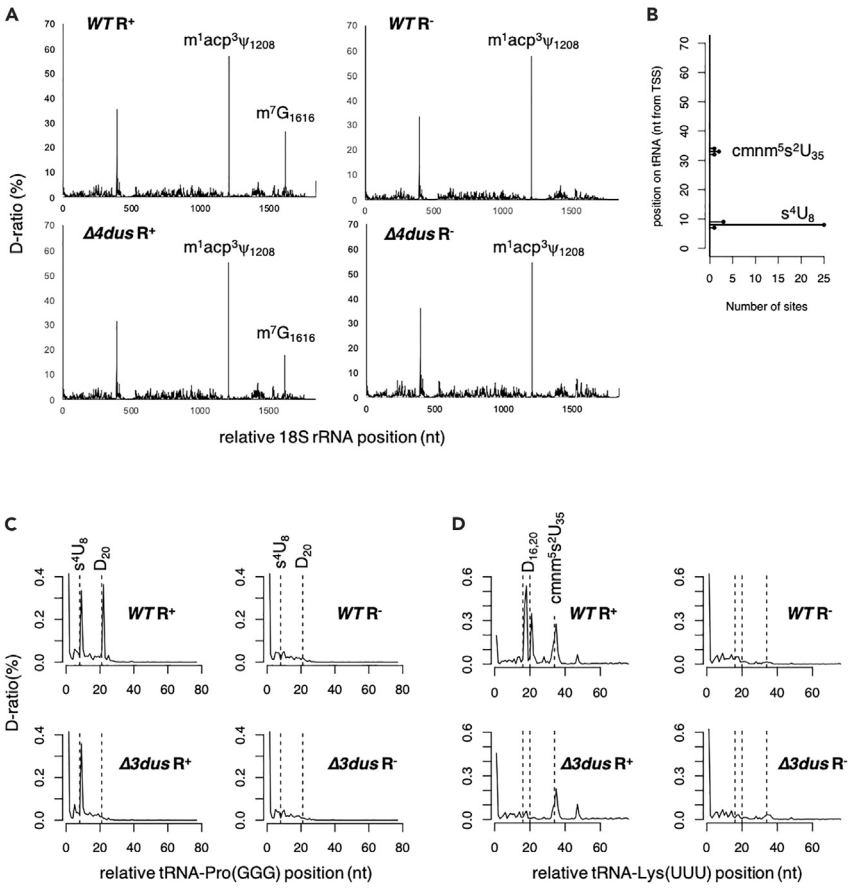

Figure 2. Application of Rho-seq beyond dihydrouridine

(A) R^+-dependent RT stop in *S. pombe* 18S rRNA. The proportion of RT-stop (D-ratio) is indicated along the 18S rRNA (SPRRNA.43). The positions 1208 and 1616 corresponding to two previously described modifications ($m^1acp^3\psi$ and m^7G, respectively) are highlighted.

(B) Distribution of the position of RT-stop sites on *E.coli* tRNAs dependent on the R^+ treatment but independent from *dus-a-b-c* genes. The Rho-seq analytical pipeline was modified to assess the significance of the treatment effect only (we computed the wald test of the treatment factor instead of the strain:treatment interaction in step 16 of the statistical analysis). The distribution of the position of sites significantly affected by the R^+ treatment peaks at position 8, a widespread s^4U modification site conserved in prokaryotes.

(C) R^+-dependent RT stop in *E. coli* tRNA-Pro(GGG). The proportion of RT-stop (D-ratio) is indicated along the Pro(GGG) tRNA (b2189). The positions 8 and 20 corresponding to two previously described modifications (S^4U and D, respectively) are highlighted.

(D) R^+-dependent RT stop in *E. coli* tRNA-Lys(UUU). The proportion of RT-stop (D-ratio) is indicated along the tRNA-Lys(UUU) (b0743). The positions 16, 20 and 35 corresponding to three previously described modifications (D, D and $cmnm^5s^2U$, respectively) are highlighted.

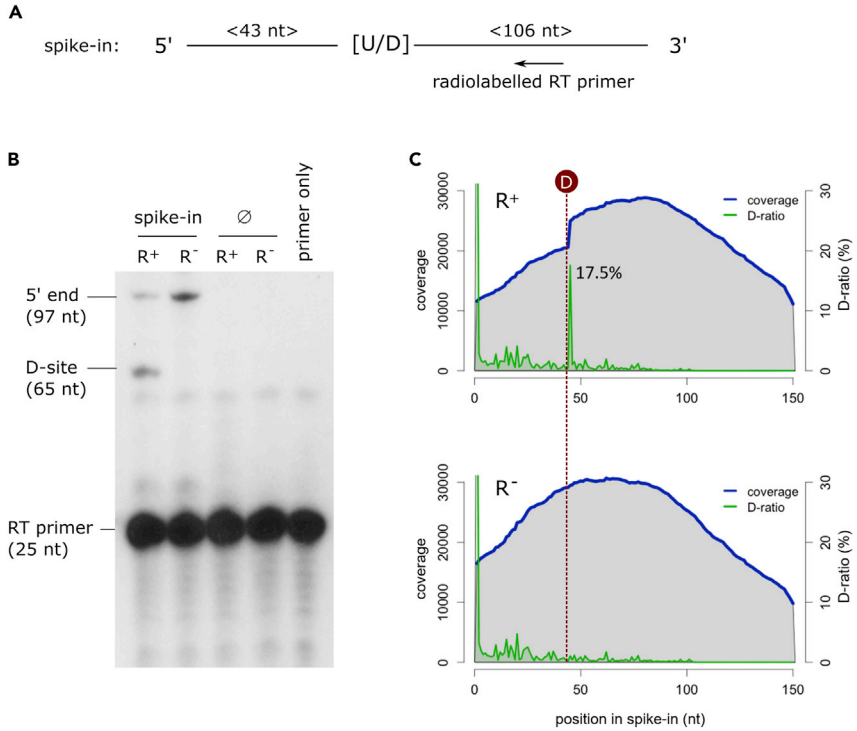

Figure 3. A dihydrouridylated spike-in internally controls Rho-seq

(A) Cartoon representation of the 150 nucleotides (nt) long spike-in. The sequence (produced by *in vitro* transcription) contains a unique uridine (U) position, that is replaced by D when the spike-in is transcribed in presence of rDTP instead of rUTP. A radiolabeled RT primer can prime reverse transcription in primer extension experiments.

(B) Primer extension assay on fully dihydrouridylated spike-in mixed with *S. pombe* total RNA before the R+ and R- treatment visualized on a 15% poly-acrylamide gel. The migration patterns of the full-length RT product (5′ end), of the premature transcription stop site at the unique D position (D-sites), and of the excess labeled RT primer are indicated.

(C) D-ratio (green line) and fragment coverage (blue line and shaded area) along the fully dihydrouridylated spike-in sequence as determined by Rho-seq. In the R+ condition, a sharp drop in coverage is observed one nucleotide downstream the unique D (position 44, highlighted with a dotted red line), that coincide with a sharp increase in D-ratio.

(contaminant-free and RIN > 9). We leave to the reader the choice of selecting the most appropriate RNA extraction method, but for yeast researchers, we recommend the classical hot phenol RNA extraction protocol (Bähler and Wise, 2017).

⚠ CRITICAL: Regardless of the RNA extraction method used, RNA handling best practice should be applied throughout the protocol: wear clean gloves, work on ice unless stated otherwise (at least until step 12) and use RNase-free water in all solution. We use DEPC-treated water but commercial RNase-free water is a good alternative.

Preparation of spike-in internal control

Timing: 1 day

Besides the (external) control conditions, an additional internal control allows to verify and calibrate the efficiency of the Rho-seq protocol. It consists of a synthetic RNA transcript generated by *in vitro* transcription of a DNA template in which only one nucleotide of the template strand is a T (Schwartz et al., 2014). *In vitro* transcription in presence of rDTP instead of rUTP ensures that 100% of the generated transcripts are dihydrouridylated on one specific position on the transcript (Figure 3A). Furthermore, controlled amount of non-dihydrouridylated spike-in (made by the same *in vitro*

transcription reaction, but in presence of rUTP instead of rDTP) can be mixed with the dihydrouridylated spike-in to provide intermediate level of D at the unique U/D position.

1. Amplify the spike-in DNA template with Phusion High-Fidelity DNA Polymerase using 20 ng of gBLOCK spike-in sequence and the spike-in forward and reverse primers according to manufacturer's instruction (without optional DMSO).
2. Run the PCR product on a 2% agarose gel at standard voltage (140 mV for 20 × 20 cm gels) and purify the 150nt-long band with QIAquick PCR Purification Kit according to manufacturer's instruction.
3. *In vitro* transcribe the RNA spike-in using ~200 ng of purified spike-in DNA template with RiboMAX™ Large Scale RNA Production Systems according to manufacturer's instruction, with the exception that the dihydrouridylated spike-in is prepared with rDTP instead of rUTP.
4. Purify synthetic RNA with ProbeQuant™ G-50 Micro according to manufacturer's instruction. Quantify using Qubit or Nanodrop and conserve at −80°C ~indefinitely.

Mixing the RNA spike-in in the input RNA samples prior to rhodamine labeling provides a useful basis to calibrate Rho-seq experiments as it connects a precise percentage of dihydrouridilation to an observed pattern of R^+-dependent RT stop (Figures 3B and 3C).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Acetic acid (CH3COOH)	Sigma-Aldrich	Cat#A6283; CAS 64-19-7
Acidic phenol:chloroform 5:1 (pH 4.3)	Sigma-Aldrich	Cat#P1944
Calcium chloride (CaCl2) powder	Sigma-Aldrich	Cat#C8106; CAS 10035-04-8
Chloroform:IAA 24:1	Sigma-Aldrich	Cat#25666
DEPC	Sigma-Aldrich	Cat#D5758; CAS 1609-47-8
Ethanol 100%	Sigma-Aldrich	CAS#64-17-5
Magnesium chloride (MgCl2) powder	Sigma-Aldrich	Cat#M826; CAS 7786-30-3
Phenol:chloroform:IAA 125:24:1	Sigma-Aldrich	Cat#77619
Potassium chloride (KCl) powder	Sigma-Aldrich	Cat#P9541; CAS 7447-40-7
Potassium hydroxide (KOH) powder	Sigma-Aldrich	Cat#8.14353; CAS 1310-58-3
Rhodamine (Rhodamine 110 chloride)	Sigma-Aldrich	Cat#83695; CAS 13558-31-1
Sodium acetate (NaAC) pH 5.2 3 M	Sigma-Aldrich	Cat#S7899; CAS 126-96-5
Sodium borohydride ($NaBH_4$) powder (Sigma 452882)	Sigma-Aldrich	Cat#452882; CAS 16940-66-2
Sodium formate (NaCOOH)	Sigma-Aldrich	Cat#71539; CAS 141-53-7
Tris-HCl pH 7.4 1 M	Teknova	Cat#T5074
Triton X-100	Sigma-Aldrich	Cat#X-100; CAS 9036-19-5
5× first strand buffer	Invitrogen	Cat#18080-093
dNTPs mix (10 mM each)	Thermo Fisher Scientific	Cat#R0194
ExoSAP-IT 40UL	Affymetrix	Cat#78250
FastAP Thermosensitive Alkaline Phosphatase	Thermo Fisher Scientific	Cat#EF0654
Fragmentation buffer	Thermo Fisher Scientific	Cat#AM8740
Murine RNase Inhibitor	New England Biolabs	Cat#M0314
NEBNext High-Fidelity 2× Master Mix	New England Biolabs	Cat#M0541
Phusion High-Fidelity DNA Polymerase	New England Biolabs	Cat#M0530S
rDTP	tebu-bio	Cat#040N-1035-1
T4 RNA Ligase 1	New England Biolabs	Cat#M0437M
TURBO DNase	Thermo Fisher Scientific	Cat#AM2238
Critical commercial assays		
AMPure XP beads (SPRI beads)	Beckman/Coulter	Cat#A63880
Bioanalyzer RNA 6000 Pico ChIP	Agilent	Cat#5067-1513
Buffer RLT (from RNeasy kit)	QIAGEN	Cat#74004
QIAquick PCR Purification Kit	QIAGEN	Cat#28106

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MyONE SILANE magnetic beads	Thermo Fisher Scientific	Cat#37002D
ProbeQuant™ G-50 Micro Columns	Sigma-Aldrich	Cat#GE28-9034-08
RiboMAX™ Large Scale RNA Production Systems	Promega	Cat#P1300
RiboMinus Eukaryote Probe Mix v2	Ambion	Cat#A15017
RiboMinus Eukaryote Kit v2	Thermo Fisher Scientific	Cat#A15020
RiboMinus Concentration module	Thermo Fisher Scientific	Cat#K155005
Deposited data		
Raw and processed Rho-seq data	Finet et al. (2022)	GSE145685
S. pombe reference genome build ASM294v2.26	pombase	www.pombase.org/data/genome_sequence_and_features/OLD/20150511/
Experimental models: Organisms/strains		
WT (*h-*)	Finet et al. (2022)	94
Δ4dus (h- dus1::nat^R dus2::gen^R dus3::hph^R dus4::ble^R)	Finet et al. (2022)	1755
Oligonucleotides		
Spike-in DNA template (T7 promoter in bold): TAA TACGACTCACTATAGGGGAGGCGAGAACACAC CACAACGAAAACGAGCAAAACCCGGTACGCAA CACAAAAGCGAACAACGCGAAAAAGGACACCG AAGCGGAAGCAAAGACAACCAACAGAAAACAA CCGCAAACAAACGGGACCAGACAACG CACCAGCAAAA	Schwartz et al. (2014); Finet et al. (2022).	N/A
Spike-in forward primer: TAATACGACTCACTATAGGGG	Finet et al. (2022).	N/A
Spike-in reverse primer: TTTTGCTGGTGCGTTG	Finet et al. (2022).	N/A
RNA 3′ adapter: /5′Phos/rArGrArUrCrGrGrA rArGrArGrCrGrUrCrGrUrG/3′ddC/	Finet et al. (2022).	N/A
RT primer: ACACGACGCTCTTCCGA	Finet et al. (2022).	N/A
DNA 3′ adapter: /5′Phos/AGATCGGAAG AGCACACGTCTG/3′ddC/	Finet et al. (2022).	N/A
Universal PCR primer: AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACG ACGCTCTTCCGATCT	Finet et al. (2022).	N/A
Barcoded PCR primer (the «NNNNNN» sequence in the barcoded PCR primer corresponds to the reverse complement of an Illumina index sequence) : CAAGCAGAAGACGGCATACGAGATNNNNNNGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	Finet et al. (2022).	N/A
Software and algorithms		
Fastqc	by Simon Andrews	bioinformatics.babraham.ac.uk/projects/fastqc
trimmomatic version 0.36	Bolger et al., 2014	usadellab.org/cms/?page=trimmomatic
bowtie2 version 2.4.4	Langmead and Salzberg, 2012	bowtie-bio.sourceforge.net/bowtie2
samtools version 1.7	Li et al., 2009	htslib.org
bedtools version 2.26.0	Quinlan and Hall, 2010	bedtools.readthedocs.io
R version 4.0.4	R Core Team, 2017	r-project.org
Rho-seq code	This study	https://zenodo.org/badge/latestdoi/76244715
Other		
2-mL Safe-lock microcentrifuge tubes	Eppendorf	0030120094
Hybond-N+ membrane	GE Healthcare	Cat#RPN1210b
Imagequant with a 520 nM filter (Cy3 channel) or similar fluorescent reader.	GE Healthcare	ImageQuant™ LAS 4000
Refrigerated centrifuge	various brands	various models
Thermomixer	various brands	various models
Thermocycler	various brands	various models
Magnetic stand for 1.5 mL microtubes.	various brands	various models
Qubit fluorometric quantification system or similar	Thermo Fisher Scientific	CAT#Q33327
Bioanalyzer or similar	N/A	N/A

MATERIALS AND EQUIPMENT

- **DEPC-treated water:** add 1 mL of DEPC to 1 L of ultra-pure (bi-distillated of milli-Q) water. Leave the water bottle with the cap loosely screwed over-night under a chemical hood (carbon dioxide will be released). Autoclave.

  Alternatives: Commercial RNAse-free water.

- **KOH 0.1 M**: 0.28 g of KOH powder in 50 mL DEPC-treated water. It can be stored at room temperature or 4°C for years.
- **6 M acetic acid:** 18 mL of 100% acetic acid in 50 mL DEPC-treated water. It can be stored at room temperature or 4°C for three months maximum.
- **Rhodamine 0.022 M:** Dissolve 80 mg of rhodamine 110 in 10 mL methanol. It can be conserved for years in small aliquots in the dark at −20°C.

  ⚠ CRITICAL: Methanol is toxic and volatile. It must be manipulated under a chemical hood.

- **pre-FNK buffer** (Engreitz et al., 2013):

Reagent	Final concentration	Amount
Tris-HCl pH 7.4 1 M	50 mM	500 µL
$MgCl_2$ 1 M	5 mM	50 µL
$CaCl_2$ 200 mM	0.6 mM	30 µL
KCl 1 M	50 mM	500 µL
Triton X-100 100%	0.01% *v/v*	1 µL
DEPC-treated water	n/a	8.82 mL
Total	**n/a**	**9.9 mL**

The pre-FNK buffer can be conserved for months at room temperature.

- **Reduction solution:**

Reagent	Final concentration	Amount
$NaBH_4$	100 mg/mL	0.1 g
KOH 0.1 M	0.01 M	100 µL
DEPC-treated water	n/a	900 µL
Total	**n/a**	**1 mL**

The reduction solution needs to be prepared freshly for each reduction reaction.

⚠ CRITICAL: The dissolution of the $NaBH_4$ powder releases fumes. Manipulate under chemical hood. To avoid over-pressure in the tubes and possible (small) explosions, we recommend preparing the 1 mL reduction solution in a 50 mL falcon.

STEP-BY-STEP METHOD DETAILS

Rhodamine labeling of dihydrouridine

Timing: 7 h

Key reagents for the rhodamine labeling:
$NaBH_4$
KOH
acetic acid
ethanol
NaAc
NaCOOH pH4
rhodamine 110 chloride
phenol-chloroform pH4.3

After the specific reduction of dihydrouridine (and potentially other modified RNA as discussed) with sodium borohydride ($NaBH_4$) treatment (Cerutti and Miller, 1967) rhodamine is incorporated in acidic conditions (Kaur et al., 2011) (Figures 1A and 4A).

1. Dihydrouridine reduction and RNA precipitation.
   a. For each condition, mix in a RNase-free 2-mL safe-lock microcentrifuge tube:

Reagent	Final concentration	Amount
total RNA from WT or *Δ4dus* strain	0.083 µg/µL	30 µg
Tris-HCl pH 7.4 1 M	40 mM final	40 mM final
in vitro transcribed spike-in RNA	8.33 pg/µL	3 ng
cold DEPC-treated water	n/a	to 360 µL

   b. For the R^+ condition (effective labeling), add 40 µL of the reduction solution to the 360 µL RNA samples to obtain a final $NaBH_4$ concentration of 10 mg/mL. For the R^- control condition (mock treatment), only add 40 µL of KOH 0.01 M.
   c. Incubate 60 min at 25°C in the dark with agitation at 750 rpm.

⚠ CRITICAL: The reduction reaction releases fumes. Manipulate under chemical hood. To avoid over-pressure in the tubes and possible (small) explosions, we recommend opening the tubes briefly after the first 10 and 20 min incubation.

   d. Add 20 µL of cold 6 M acetic acid to stop the reaction by lowering the pH to 4–5.

Note: Be careful when adding the acetic acid as it rapidly reacts in contact to the RNA solution and froth (Figures 4B and 4C).

   e. Precipitate the RNA by adding 1050 µL of cold (−20°C) 100% EtOH and 42 µL of 3 M NaAC pH 5.2.
   f. Vortex 20 s.
   g. Freeze 30 min at −80°C to help RNA precipitate.
   h. Centrifuge 10 min at 18 000 g, 4°C.
   i. Discard supernatant by pipetting and add 420 µL of cold 70% EtOH.

⚠ CRITICAL: Do not resuspend nor vortex to avoid losing the (mostly invisible) RNA pellet.

   j. Spin 1 min at 18,000 g, 4°C.
   k. Aspirate most supernatant, spin 5 s and remove rest of liquid with pipette.
   l. Air dry 10 min at room temperature.
   m. Resuspend with 5 µL of DEPC-treated water and 85 µL of 0.1 M NaCOOH pH 4.0 by heating 7 min at 65°C at 550 rpm and pipetting.

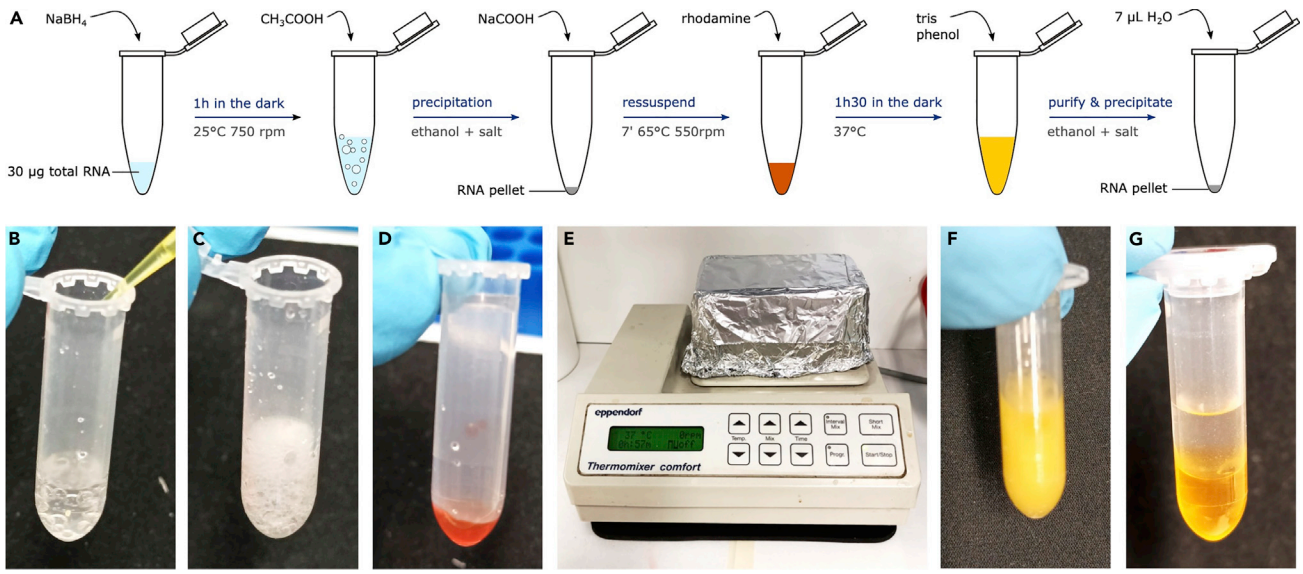

Figure 4. Rhodamine labeling workflow

(A) Detailed workflow of the rhodamine labeling protocol. See main text for details.

(B and C) Upon the addition of acetic acid to the $NaBH_4$-treated samples (**b,**), the solution reacts rapidly and froth (C).

(D) The samples take an orange tint after the addition of rhodamine-110 in acidic conditions.

(E) Covering a thermomixer in aluminum foil allows the incubation of the samples with rhodamine in the dark.

(F) Adjusting the pH with tris and phenol turn the samples yellow.

(G) Following phenol RNA purification, unincorporated rhodamine separates in the lower, organic phase, while RNA (including rhodamine-labeled RNA) separates in the upper, aqueous phase.

Note: To resuspend with a salt solution at pH 3 instead of pH 4 in step [1.m] enhances the efficiency of rhodamine incorporation and allows to use 0.1 M sodium acetate instead of sodium formate. However, it leads to a higher degree of RNA degradation (Figure S1).

n. Vortex, spin and proceed immediately to the next step.

2. Rhodamine incorporation in reduced dihydrouridine.
   a. Add 10 µL of rhodamine 0.022 M (Figure 4D).
   b. Incubate 90 min at 37°C in the dark (Figure 4E).
   c. Add 17 µL Tris-HCl 800 mM pH 8.5 to adjust the pH at 7.5.
   d. Add 433 µL DEPC-treated water to adjust the volume to 550 µL.
   e. Phenol-purify with 550 µL of acidic (pH 4.3) phenol–chloroform, vortex 20 s (Figure 4F).

⚠ CRITICAL: Phenol is toxic and volatile. It must be manipulated under a chemical hood.

   f. Centrifuge 15 min at 18,000 g, 4°C.
   g. Transfer 500 µL of the water phase in a new RNase-free 2-mL microcentrifuge tube (Figure 4G).
   h. Precipitate the RNA by adding 1500 µL of cold (−20°C-) 100% EtOH and 50 µL of 3 M NaAC pH 5.2.
   i. Vortex 20 s.
   j. Freeze 30 min at −80°C.
   k. Centrifuge 10 min at 18,000 g, 4°C.
   l. Discard supernatant and wash carefully with 500 µL of 70% EtOH (4°C).
   m. Spin 1 min at 18,000 g, 4°C.
   n. Aspirate most supernatant, spin 5 s and remove the rest of liquid with pipette.

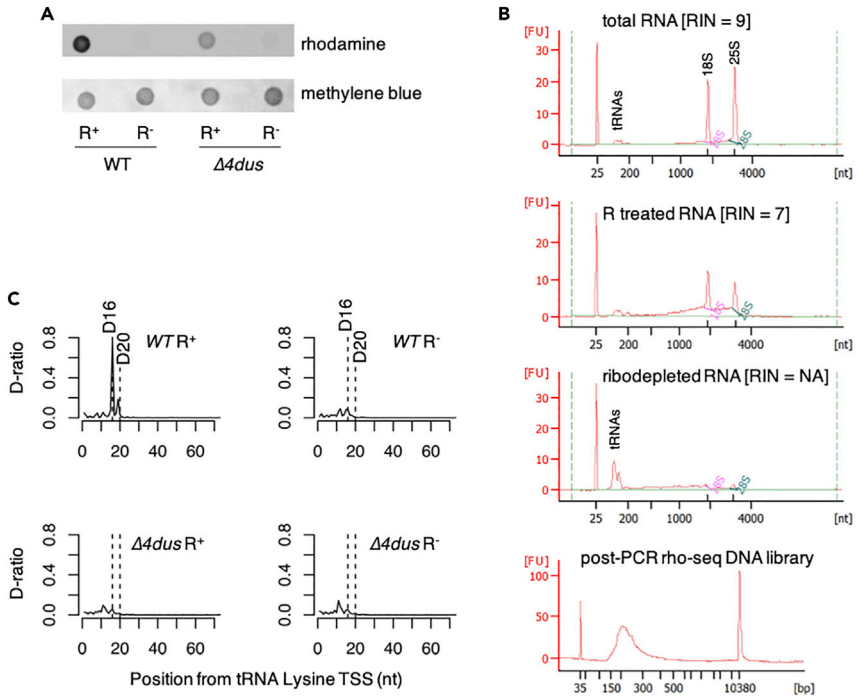

Figure 5. Expected results

(A) Comparative rhodamine intensity signal measured at 520 nm in a dot blot assay after rhodamine labeling (R^+) or mock treatment (R^-) with *WT* and *Δ4dus* total RNA. Methylene blue staining serves as loading control.

(B) Typical size distribution of samples at various step of the Rho-seq procedure as analyzed using bioanalyzer RNA pico for RNA samples or DNA high-sensitivity (HS) chips for cDNA samples. From top to bottom: total RNA before rhodamine labeling, total RNA after rhodamine labeling (the acidic treatment causes RNA degradation), ribodepleted RNA, and PCR-amplified rho-seq cDNA library. RIN = RNA integrity number.

(C) Example Rho-seq results for a Lysine tRNA (SPMITTRNALYS.01). High D-ratio can be observed one nucleotide upstream of the known D-sites at position 16 and 20.

o. Air dry 10 min at RT.

p. Resuspend in 7 μL DEPC-treated water by heating 7 min at 65°C in a thermomixer with shaking at 550 rpm.

q. Vortex and spin.

r. Dilute 1 μL of sample in 10 μL DEPC-treated water and quantify it at the nanodrop or Qubit. The expected concentration of the diluted sample is between 0.3 and 0.5 μg/μL. Troubleshooting 1.

Pause point: Samples can be stored at −80°C for years.

3. Verification of rhodamine incorporation by dot blot assay.

a. Spot a drop (4 μL) of sample RNA (diluted between 0.2 and 1 μg/uL) on a Hybond-N+ membrane and add control drops (pure DEPC-treated water, Rhodamine 0.022 M, methanol) (Figure 5A).

b. Measure rhodamine incorporation at 520 nm (Cy3 channel) in a fluorescent reader (Image-quant or similar). Calculate signal intensity with ImageJ (Schneider et al., 2012) for each drop. To normalize the intensities, subtract the signal of the DEPC-treated water drop (background) from all other values. Then, scale to the signal of the pure Rhodamine 0.022 M drop (that is set at 100%). Troubleshooting 2.

RNA ribodepletion and sample concentration

Timing: 2 h

As ribosomal RNA (rRNA) accounts for a large majority of the transcriptome, efficient ribodepletion allows to increase the ultimate read coverage on mRNA and ncRNA (other than rRNA) genes. The following steps describe the use of the Ribominus depletion system but other kits can be used.

4. RNA Ribodepletion.
   a. Heat 2× Hybridization Buffer 10 min at 50°C in a 2-mL heat block to bring salts into solution and thaw the Ribominus Eukaryote Probe Mix v2 on ice. Set heat blocks to 37°C and 70°C.
   b. Prepare 200 µL/sample of 1× Hybridization buffer by diluting 2× Hybridization buffer in RNase-free water.
   c. Following this order, mix.

Reagent	Amount
pre-heated 2× Hybridization Buffer	50 µL
RiboMinus Eukaryote Probe Mix v2	2 µL
total RNA	4 µg
DEPC-treated water	to 100 µL

   d. Mix by gentle vortexing and incubate at 70°C for 10 min.
   e. Immediately transfer the tube to 37°C for 20 min.
   f. Wash RiboMinus magnetic beads (add liquid, place the tubes on a magnetic rack until the solution clears, then remove supernatant) twice with 1 mL of DEPC-treated water.
   g. Resuspend the beads in 200 µL 1× Hybridization Buffer and incubate in the 37°C heat block for a minimum of 5 min or until the 20-min incubation of the RNA/probe mix is complete.
   h. Briefly centrifuge the RNA/probe mix.
   i. Transfer the 100 µL RNA/probe mix to the 200 µL prepared beads from step [4.g] and mix well by pipetting up and down.
   j. Incubate at 37°C for 15 min.
   k. Briefly centrifuge and place it on a magnetic stand until the solution clears.
   l. Transfer the 300 µL supernatant containing the rRNA-depleted RNA to an RNase-free 2-mL microcentrifuge tube.
5. Sample concentration.
   a. Before using Wash Buffer (W5) for the first time, add 6 mL 96–100% ethanol to 1.5 mL W5 included in the kit.
   b. Add 300 µL Binding Buffer L3 (1 volume) and 900 µL 100% ethanol (3 volumes) for a final concentration of 60% ethanol.

⚠ CRITICAL: To avoid the loss of RNAs < 200 nt (such as tRNAs) during the concentration step, the binding of RNA is performed with 60% ethanol to retain all RNA species.

   c. Mix by vortexing.
   d. Transfer 600 µL of the sample to a spin column in a collection tube (both supplied in the RiboMinus™ Concentration Module kit).
   e. Centrifuge 1 min at 12,000 g and discard flow-through.
   f. Repeat steps [d-e] with the remaining sample (in the same column).
   g. Place the column into a clean wash tube supplied in the kit.
   h. Wash the column by adding 600 µL Wash Buffer (W5) prepared with ethanol.
   i. Centrifuge the column 1 min at 12,000 g and discard flow-through.
   j. Centrifuge at maximum speed for 3 min to remove any residual W5 buffer and place the column in a clean 1.5-mL recovery tube.

k. Add 16 μL RNase-free water to the center of the column and incubate 1 min at room temperature.
l. Centrifuge at maximum speed for 1 min. Keep the flowthrough.

Pause point: Samples can be stored at −80°C.

6. Verification of ribodepletion.
   a. Quantify RNA samples using Qubit RNA HS assay or similar according to manufacturer's instructions. Typical yield is in the 10–30 ng/μL range. A minimum of 100 ng is required to proceed to the rho-seq library preparation.

⚠ CRITICAL: At this step, it is important to measure RNA concentration with fluorescent assay (Qubit and similar) and not with photometric assays (nanodrop) as the later tends to report less reliable measurements.

   b. Run total RNA (input from step 1), treated RNA (R+/R- not ribodepleted, from step 30) and ribodepleted samples in a RNA 6000 Pico bioanalyzer chip according to the manufacturer's instructions (Figure 5B). Troubleshooting 3. Troubleshooting 4.

Library preparation

Timing: 2 days

Key reagents for the Library preparation:
Fragmentation buffer
MyONE SILANE magnetic beads
AMPure XP beads (SPRI beads)
Ethanol
TURBO DNase
Murine RNase Inhibitor
FastAP Thermosensitive Alkaline Phosphatase
T4 RNA Ligase 1
PEG 8000
5× first strand buffer
FastAP Thermosensitive Alkaline Phosphatase
ExoSAP-IT 40UL
NEBNext High-Fidelity 2× Master Mix

The library preparation starts with the fragmentation of the ribodepleted RNA. Then, the 3′ of the RNA fragments are ligated to an RNA adaptor that hybridizes to a universal RT primer to allow reverse transcription. Because it is sensitive to rhodaminized nucleotides, RT converts the initial presence of D into a quantifiable signal and is therefore a central step of this protocol. Indeed, while D barely affects RT (Motorin et al., 2007), we found that the addition of the bulky rhodamine moiety onto the D residue after effective rhodamine labeling (R^+) effectively blocks reverse transcriptase and leads to specific RT drop-off (Finet et al., 2022). In consequence, the shorter, truncated cDNA fragments generated have 3′-ends that reflects the presence of D one nucleotide downstream on the template strand (Figure 1B).

Critically, the information on the 3′-end position needs to be preserved during second-strand synthesis. This requirement precludes the application of the common RNAse-H-based method used to generate second-strand RNA primers in Illumina sequencing chemistry (Sultan et al., 2012).

Instead, direct ligation of a second adaptor in 3′ of the cDNA allows to preserve its end during the PCR amplification and paired-end sequencing of the library (Engreitz et al., 2013; Agarwal et al., 2015) (Figure S2).

⚠ CRITICAL: Throughout library preparation, there are multiple purification steps using magnetic beads. In that context, to "wash the beads" means to add a liquid (volume and nature are indicated) to the beads, place the tubes on a magnetic rack until the solution clears, then remove supernatant by pipetting while leaving the beads in the tube.

7. Fragmentation.
   a. Bring 90–125 ng of ribodepleted RNA to a 9 µL volume with DEPC-treated water.
   b. Add 1 µL of 10× Fragmentation Buffer.
   c. Mix by vortexing, spin briefly, and incubate at 70°C for 15 min in a heating block.
   d. Add 2 µL of the Stop Solution and incubate 30 s at room temperature.
   e. Spin sample briefly then place it back on ice.

8. Elution.
   a. Resuspend the MyONE SILANE magnetic beads by thorough vortexing and aliquot out 15 µL in a 1.5 mL nucleases-free tube.
   b. Place tube on a magnetic rack and wait 30 s.
   c. Discard supernatant and wash the beads with 15 µL RLT Buffer.
   d. Resuspend bead in 42 µL RLT Buffer and add the beads to sample.
   e. Add 54 µL 100% ethanol and mix well by pipetting (about 10 up and downs).
   f. Incubate for 2 min at room temperature.
   g. Place tube on magnetic track and wait 1 min, then discard supernatant.
   h. Wash twice by resuspending beads in 150 µL of 70% ethanol.

   ⚠ CRITICAL: Always work with a fresh stock of 70% ethanol.

   i. Carefully remove all remaining 70% ethanol and dry beads on magnetic rack at room temperature for 5 min. Troubleshooting 5.
   j. Resuspend beads in 32 µL DEPC-treated water (4°C), place tube on a magnetic rack, wait 30 s or until solution clears and transfer eluate to a nucleases-free PCR tube (keep on ice).

9. DNase digestion and RNA preparation.
   a. Preheat a thermal cycler at 37°C (lid at 105°C).
   b. Freshly prepare FNK buffer by adding 1 µL DTT 1 M in 99 µL pre-FNK buffer.
   c. Combine the sample with 18 µL of a master mix containing:

Reagent	Amount
5× FNK Buffer	10 µL
Murine RNase Inhibitor	1 µL
FastAP Thermosensitive Alkaline Phosphatase	3 µL
TURBO DNase	1 µL
DEPC-treated water	3 µL

   d. Mix well and incubate at 37°C for 30 min.
   e. Thaw 10× NEB Ligase 1 Buffer, ATP and PEG 8000 (−20°C) at room temperature for the next part of the protocol.
   f. Transfer the sample into a new 1.5 mL microcentrifuge tube.
   g. Resuspend the MyONE SILANE magnetic beads by thorough vortexing and aliquot out 15 µL in a 1.5 mL nucleases-free tube.

h. Place tube on a magnetic rack and wait 30 s.
i. Discard supernatant and wash beads with 15 µL RLT Buffer.
j. Resuspend bead in 150 µL RLT Buffer and add to sample.
k. Add 50 µL 100% ethanol and mix well by pipet.
l. Incubate for 2 min at room temperature.
m. Place tube on magnetic track and wait 1 min, then discard supernatant.
n. Wash twice by resuspending beads in 150 µL 70% ethanol.
o. Carefully remove all remaining 70% ethanol and dry beads on magnetic rack for minimum 5 min (until dry).
p. Resuspend beads in 6 µL DEPC-treated water (4°C)), place tube on a magnetic rack, wait 30 s or until solution clears and transfer eluate to a 1.5 mL nucleases-free tube (keep on ice).

10. First ligation.
    a. Add 0.5 µL of 40 µM (= 20 pmol) RNA adapter ressuspended in DEPC-treated water.
    b. Denature at 70°C for 2 min and then transfer immediately to ice.
    c. Prepare the 13.6 µL/sample ligation master mix at room temperature by mixing the following:

Reagent	Amount
10× NEB Ligase 1 Buffer	2 µL
DMSO 100%	1.8 µL
ATP (100 mM)	0.2 µL
PEG 8000 (50%)	8 µL
Murine RNase Inhibitor	0.3 µL
T4 RNA Ligase 1 (30 U/µL)	1.3 µL

⚠ CRITICAL: PEG 8000 is very viscous and should be pipetted using truncated tips. Prepare 25% more of the master mix to account for pipetting error. Room temperature is required at this step to avoid DMSO precipitation.

    d. Close cap, mix by flicking and spinning down the tube three times.
    e. Add 13.6 µL to each sample and mix by flicking vigorously and spinning down tubes three times.
    f. Incubate at 25°C for 90 min.
    g. Resuspend the MyONE SILANE magnetic beads by thorough vortexing and aliquot out 15 µL in a 1.5 mL nucleases-free tube.
    h. Place tube on a magnetic rack and wait 30 s.
    i. Discard supernatant and wash beads with 15 µL RLT Buffer.
    j. Resuspend bead in 61 µL RLT Buffer and add to sample.
    k. Add 65 µL 100% ethanol and mix well by pipetting.
    l. Incubate for 2 min at room temperature.
    m. Place tube on magnetic track and wait 1 min, then discard supernatant.
    n. Wash twice by resuspending beads in 150 µL 70% ethanol.
    o. Carefully remove all remaining 70% ethanol and dry beads on magnetic rack for minimum 5 min (until dry).
    p. Resuspend beads in 5 µL cold DEPC-treated water (4°C), place tube on a magnetic rack, wait 30 s or until solution clears and transfer eluate to a 1.5 mL nucleases-free tube (keep on ice). Proceed directly to the next step.

11. Reverse transcription – first strand cDNA synthesis.
    a. Add 1 µL of 10 µM RT primer (10 pmol) to each 5 µL sample.
    b. Denature at 70°C for 2 min, then immediately transfer to ice to cool down.

c. Add 4 μL of reverse transcription master mix:

Reagent	Amount
1 mM dNTPs stock	1.5 μL
5× first strand buffer	2.0 μL
SuperScript III Reverse Transcriptase	0.5 μL

d. Quickly mix by pipetting and transfer to a preheated 50°C heating block.
e. Incubate at 50°C for 60 min.
f. Remove samples from heating block and transfer on ice, wait 1 min, spin and transfer into a 1.5 mL nucleases-free tube.
g. Digest excess RT primer by adding 2 μL of ExoSAP-IT.
h. Mix and incubate at 37°C for 4 min.
i. Inactivate the ExoSAP-IT by incubating at 80°C for 1 min.
j. Snap cool on ice and spin down the tubes.

12. cDNA clean-up and RNA degradation.
a. Add 10.95 μL nucleases-free water to each 12 μL sample.
b. Degrade RNA by adding 2.55 μL 1 M NaOH (100 mM final concentration).
c. Incubation at 70°C for 10 min.
d. Thaw 10× NEB Ligase 1 Buffer, ATP and PEG 8000 at room temperature for the next part of the protocol.
e. Cool samples on ice, spin, and neutralize solution by adding 2.55 μL of 1 M acetic acid.
f. Resuspend the MyONE SILANE magnetic beads by thorough vortexing and aliquot out 12 μL in a 1.5 mL nucleases-free tube.
g. Place tube on a magnetic rack and wait 30 s.
h. Discard supernatant and wash beads in 12 μL RLT Buffer.
i. Resuspend bead in 75 μL RLT Buffer and add them to the sample.
j. Add 65 μL 100% ethanol and mix well by pipetting.
k. Incubate for 2 min at room temperature.
l. Place tube on magnetic track and wait 1 min, then discard supernatant.
m. Wash twice by resuspending beads in 150 μL 70% ethanol.
n. Carefully remove all remaining 70% ethanol and dry beads on magnetic rack for minimum 5 min (until dry).
o. Resuspend beads in 5.5 μL cold DEPC-treated water (4°C), place tube on a magnetic rack, wait 30 s or until solution clears and transfer eluate to a new 1.5 mL nucleases-free tube.

13. Second ligation.
a. Add 0.5 μL of 80 μM (= 40 pmol) DNA adapter.
b. Denature at 75°C for 2 min and then transfer immediately to ice.
c. Prepare the 14.1 μL/sample ligation master mix (prepare 25% more to account for pipetting error with PEG 8000) by mixing the following:

Reagent	Amount
10× NEB Ligase 1 Buffer	2.0 μL
DMSO (100%)	0.8 μL
ATP (100 mM)	0.2 μL
PEG 8000 (50%)	9.5 μL
T4 RNA Ligase 1 (30 U/μL)	1.6

d. Close cap, mix by flicking and spinning down tube three times.

e. Add 14.1 μL to each sample and mix by flicking vigorously and spinning down tubes three times.

Note: From this step, working on ice is no longer necessary.

f. Incubate at 25°C overnight in a thermomixer and mix by shaking 1 min at 1,000 rpm every 30 min.

Pause point: Overnight incubation is always a good time to make a break. In case your thermomixer does not have the interval mix option, continuous gentle shaking (750 rpm) is a reasonable alternative.

g. Thaw NEBNext High-Fidelity 2× Master Mix on ice.
h. Resuspend the MyONE SILANE magnetic beads by thorough vortexing and aliquot out 5 μL in a 1.5 mL nucleases-free tube.
i. Place tube on a magnetic rack and wait 30 s.
j. Discard supernatant and wash beads in 5 μL RLT Buffer.
k. Resuspend bead in 61 μL RLT Buffer and add to sample.
l. Add 55 μL 100% ethanol and mix well by pipetting.
m. Incubate for 2 min at room temperature.
n. Place tube on magnetic track and wait 1 min, then discard supernatant.
o. Wash twice by resuspending beads in 150 μL 70% ethanol.
p. Carefully remove all remaining 70% ethanol and dry beads on magnetic rack for minimum 5 min (until dry).
q. Resuspend beads in 25 μL water, place tube on a magnetic rack, wait 30 s or until solution clears and transfer 21 μL of eluate to PCR tubes.

14. PCR enrichment and clean-up.
    a. Set up 50 μL PCR reactions:

Reagent	Amount
cDNA (from previous step)	21 μL
Barcoded PCR primer (25 μM)	2 μL
Universal PCR primer (25 μM)	2 μL
NEBNext High-Fidelity 2× Master Mix	25 μL

b. Run the following PCR program:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	5 cycles
Annealing	67°C	30 s	
Extension	72°C	30 s	
Denaturation	98	10 s	13 cycles
Annealing and Extension	72	30 s	
Final extension	72°C	1 min	1
Hold	4°C	forever	

c. Bring the AMPure XP beads at room temperature for 30 min.
d. Transfer the amplified cDNA into a 1.5 mL tube.
e. Resuspend the beads by thorough vortexing.

f. Add 60 μL AMPure XP beads (= 1.2 × volume).

⚠ CRITICAL: Pipet carefully and slowly to make sure to have the correct AMPure XP volume as the beads solution is quite viscous.

g. Mix well by pipetting and wait 5 min.
h. Place on magnet and wait 3 min.
i. Remove and discard supernatant.
j. Wash beads twice in 100 μL freshly-prepared 70% ethanol.
k. Open the tubes and dry beads at 37°C for 7 min (or until dry) in a heat block.
l. Add 40 μL water but do not remove from beads.
m. Add 50 μL AMPure XP beads (= 1.1 × volume).
n. Mix well by pipetting and wait 5 min.
o. Place on magnet and wait 3 min.
p. Remove and discard supernatant.
q. Wash beads twice in 100 μL 70% ethanol.
r. Open the tubes and dry beads at 37°C for 7 min (or until dry) in a heat block.
s. Resuspend beads in 25 μL water, place tube on a magnetic rack, wait 30 s or until solution clears and transfer 23 μL of the eluate into a new 1.5 mL tube.

15. Verification of the cDNA library.
    a. Measure library concentration with Qubit fluorometric quantitation dsDNA HS Assay Kit.
    b. Examine DNA fragment sizes using the High-Sensitivity DNA Bioanalyzer kit (Figure 5B). Troubleshooting 6.
    c. Sequence library on Illumina sequencer (HiSeq, Nextseq or similar) with paired end reads (50 bp each).

⚠ CRITICAL: Only paired-end sequencing allows to properly estimate the fragment coverage, a critical metric for the identification of dihydrouridylated sites. Reads longer than 50 bp are usually not worth the extra-cost but can be chosen if needed (*e.g.*, when sequencing in a pool of other libraries). Sequencing depth, however, is a critical parameter. We recommend a minimal depth of coverage of 100× for *S. pombe* (at least 30 millions paired-end reads per library).

EXPECTED OUTCOMES

Incorporation of the rhodamine fluorophore should be efficient in the WT R+ condition, largely inefficient in the R- conditions, and intermediate in the dihydrouridine-free *Δ4dus* R+ condition (Figure 5A). The remaining signal in the *Δ4dus* R+ condition can be attributed to the non-dus modifications sensitive to the R treatment such as m^7G. Note that the acidic treatment during R^+ and R^- rhodamine labeling causes mild RNA degradation (Figure 5B).

After Rho-seq library preparation and sequencing, it is expected to observe a R-dependent RT arrest at the position 62 in the dihydrouridylated spike-in (Figure 3C). In addition, robust R-dependent and dus-dependent RT arrest at known D-sites position with the D-loop of tRNAs, typically at the positions 16 and 20 (Figure 5C). Finally, D-sites can be found on specific mRNA in eukaryotes, including tubulin-encoding mRNAs (Finet et al., 2022).

QUANTIFICATION AND STATISTICAL ANALYSIS

The main challenge of the analysis of Rho-seq data is to find transcriptomic positions where the rate of RT stop is significantly increased in the WT R^+ condition while controlling for the background rate of RT stop in the other conditions. A step-by-step methodology to infer D-sites from Rho-seq raw sequencing data is described below.

Reads pre-processing and alignment

All following steps must be applied to each of the Rho-seq libraries referred hereafter as <${sample}>. To allow users to easily try and test the pipeline, a toy example containing only reads that map to the spike-in sequence is available on www.github.com/cyaguesa/Rho-seq/tree/master/toy_exemple.

1. Verify R1 and R2 read quality using FASTQC.

```
fastqc -t 4 ${sample}.R1.fastq.gz

fastqc -t 4 ${sample}.R2.fastq.gz
```

2. Remove low-quality reads and trim adapters using trimmomatic.

```
trimmomatic PE -threads 4 -phred33 ${sample}.R1.fastq.gz ${sample}.R2.fastq.gz ${sample}.R1.filtered.paired.gz ${sample}.R1.filtered.unpaired.gz ${sample}.R2.filtered.paired.gz ${sample}.R2.filtered.unpaired.gz ILLUMINACLIP:adapter_file.fa:2:30:10 SLIDINGWINDOW:4:15 MINLEN:20
```

3. Verify trimmed read quality using FASTQC.

```
fastqc -t 4 ${sample}.R1.filtered.paired.gz

fastqc -t 4 ${sample}.R2.filtered.paired.gz
```

4. Align reads to an indexed reference transcriptome <$reference_index> including the spike-in sequence (a pre-indexed spike-in sequence is available on www.github.com/cyaguesa/Rho-seq/tree/master/toy_exemple).

```
bowtie2 -x $reference_index -1 ${sample}.R1.filtered.paired.gz -2 ${sample}.R1.filtered.paired.gz –no-discordant –no-mixed –phred33 -p 4 –no-unal | \

samtools view -uS - > ${sample}_aligned.bam
```

Note: Aligning to a reference transcriptome makes the downstream analysis more straightforward since there are no spliced alignments to deal with and only one strand to consider. However, it makes the detection of D on introns or unannotated features impossible. In *S.pombe*, we did not detect D on any intron despite considering the full the genome in our original analysis. Therefore, a transcriptome-based analysis, as described in details in this protocol, is appropriate. We acknowledge that the situation may differ in other species for which genome-based analysis can be preferred. In those cases, we refer the reader to the original Rho-seq analysis code available on github: www.github.com/cyaguesa/Rho-seq.

5. Sort and index the alignment (.bam) file using samtools.

```
samtools sort ${sample}_aligned.bam -o ${sample}_aligned_sorted.bam

samtools index ${sample}_aligned_sorted.bam
```

Computation of RT stop and fragment coverage

After trimming and mapping the sequenced paired-end reads to a reference sequence, three basic metrics are computed for every transcriptomic position and every condition:

- The fragment coverage, i.e., the number of fragments covering a transcriptomic position. Its calculation requires the computational extension of read pairs from their leftmost coordinate to their rightmost coordinate in transcriptome space.
- The number of RT stop events per position reflected by the number of R2 reads starting in that position. This metric is directly impacted by the rhodamine labeling of D residues, which can force RT to stop prematurely.
- The D-ratio, which is the ratio between the number of RT stops and the fragment coverage at given position.

6. Split mapped reads according to strand and orientation. Here we only process the forward strand.

```
samtools view -b -f 82 -F 256 ${sample}_aligned_sorted.bam > ${sample}.fwd1.bam && \
samtools index ${sample}.fwd1.bam
samtools view -b -f 130 -F 272 ${sample}_aligned_sorted.bam > ${sample}.fwd2.bam && \
samtools index ${sample}.fwd2.bam
samtools merge ${sample}.fwd.bam ${sample}.fwd1.bam ${sample}.fwd2.bam && \
samtools index ${sample}.fwd.bam && \
rm ${sample}.fwd1.bam && \
rm ${sample}.fwd2.bam
```

7. Calculate the number of RT stop events per position, i.e., the number of R2 reads ending in that position.

```
bedtools genomecov -ibam ${sample}.fwd.bam -strand + -d -5 | \
sort -k 1,1 -k2,2n -r > ${sample}.fwd.RTstop
```

8. Calculate the extended fragment coverage, i.e., the read coverage from the leftmost coordinate to the rightmost coordinate of a read pair.

```
samtools sort -l 0 -@ 1 -m 3G -n ${sample}.fwd.bam | \
bedtools bamtobed -i stdin -bedpe | \
awk 'BEGIN {OFS="\t"};{m=$2;M=0;for(i=2;i<=6;i++)if((i != 4)) {if(($i<m))m=$i;if(($i>M))
M=$i};print $1,m,M}' | \
sort-bed -max-mem 5G - | \
bedtools genomecov -i stdin -d -g $reference | \
sort -k 1,1 -k2,2n -r > ${sample}.fwd.coverage
```

Note: The final <sort> function ensures that each entry of the coverage file matches with the corresponding line of the RTstop file.

Combination and pre-filtering

Due to the very high number of positions to be tested for the presence of D throughout the transcriptome, multiple testing correction is critical to keep the false positive rate under control. However, although necessary, p-values correction with the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) also leads to a loss of statistical power that is proportional to the number of negative sites tested. To mitigate this loss, we took inspiration from a pre-filtering strategy previously applied to the differential expression analysis of RNA-seq data (Bourgon et al., 2010; Love et al., 2014) and implemented the following independent criteria to remove sites unlikely to yield positive results prior to testing them:

- The sites that are not preceded by an annotated U/T.
- The sites for which we have insufficient observations (fragment coverage > x).
- The sites that do not stop reverse transcription across conditions (overall mean D-ratio < y).
- The sites for which the proportion of RT stop remains invariable across conditions (standard deviation of the mean D-ratio < z).

x, y and z are arbitrary thresholds, but can be rationally estimated for each Rho-seq experiments based on the results obtained from the dihydrouridylated spike-in. For instance, we observed that robust, yet incomplete spike-in dihydrouridilation (50% D/U) results in a D-ratio of about 7.2% (Figure S3A). In such a setting, repeated down-sampling of the Rho-seq reads reveals that D-ratio estimation becomes more variable as the fragment coverage decreases (Figure S3B). Therefore, we set the threshold for x (minimal fragment coverage) to 50 in order to ensure that sufficient observation will support robust D-ratio estimate. Similarly, we set the threshold for y (minimal mean D-ratio across conditions) and z (minimal standard deviation of D-ratio) to 1.8 and 0.02 respectively considering that hypothetic D-sites robustly dihydrouridylated can be expected to show a D-ratio of at least 7.2% in the WT R^+ condition and close to 0–1% in the three other control conditions.

9. Merge all coverage and RTstop files across conditions and calculate the average fragment coverage. In this example, it assumes that only two conditions are processed together.

```
paste *.fwd.coverage *.fwd.RTstop |

awk 'BEGIN {OFS=''\t''};{print $1,$2,$3,$6,$9,$12,($3+$6)/2}' > combined.coverage
```

10. Filter out sites with average fragment coverage < $x and calculate D-ratio. A pseudocount of 1 is added to the numerator and denominator to avoid division by zero.

```
awk 'BEGIN {OFS=''\t''}; ($7 > $x) {print $0,($5+1)/($3+1),($6+1)/($4+1)}' combined.
coverage > combined.Dratio
```

11. Calculate average D-ratio and filter out sites with average D-ratio < $y.

```
awk 'BEGIN {OFS=''\t''}; (($8+$9)/2 > $y){print $0,($8+$9)/2}' combined.Dratio > combined.
Dratio.filtered
```

12. Filter out sites with D-ratio standard deviation < $z.

```
awk 'BEGIN {OFS=''\t''}; sqrt((((($8-$10)*($8-$10))+(($9-$10)*($9-$10)))*/2) > $Z){print
$0}' combined.Dratio.filtered > combined.Dratio.filtered2
```

13. Find upstream nucleotide in the reference transcriptome file and keep only sites with a T/U upstream. Note that contrary to the coverage files, the intermediary bed file is zero-based for the start coordinate.

```
awk 'BEGIN {OFS=''\t''}; {print $1, $2-2, $2-1}' combined.Dratio.filtered2 > filtered2.bed

bedtools getfasta -tab -fi reference.fasta -bed filtered2.bed -fo filtered2.seq

paste combined.Dratio.filtered2 filtered2.seq | awk 'BEGIN {OFS=''\t''}; ($14 == ''T'') {print
$0}' > combined.Dratio.filtered3
```

The filtered file should have column like this:

```
chrom | position | cov#1... cov#n | stop#1... stop#n | average_cov | Dratio#1... Dratio#n | average_
Dratio | chrom | (position-2) | (position-1) | upstream_sequence
```

Statistical analysis

The D-ratio, expressed as percentage, is a metric that allows the comparison of the proportion of RT stopping at a given position with a single number. For instance, on a fully dihydrouridylated RNA spike-in, we observed 17.5% of D-ratio one nucleotide downstream the unique D in the R^+ condition, but only 1% in the R- condition, clearly highlighting the increased propensity of D to cause RT-stop after the R^+ treatment (Figure 3C). However, one drawback of the D-ratio expressed as a percentage is that it loses information about the depth of coverage – the number of total observations of RT stops or readthrough at a position, which is one of the key factors to confidently call a site dihydrouridylated.

In consequence, D calling in Rho-seq is based on the raw counts of RT stops and fragment coverage, or, in other words, on the D-ratio expressed as a fraction of two integers. Like any random variable expressed as a binary outcome of repeated experiments (RT either stops or passes through a given position), the D-ratio as a fraction is best modelized with a binomial model. Simple binomial test, however, are ill-suited to accommodate the complexity of the multifactorial experimental design of Rho-seq. Instead, Rho-seq propose to use the flexible framework of a generalized linear model of the binomial family (Nelder and Wedderburn, 1972). In the model, for each position, the outcome (D-ratio) is explained by the sum of three factors: the treatment effect (R+ or R-), the strain effect (WT or dihydrouridine-free *Δ4dus*) and their interaction (Figure 1C). Additional factors, such as batch effects if the replicate samples were not processed simultaneously, can readily be added into the model.

In the context of Rho-seq, putative D-sites are sites in which the D-ratio is significantly higher in the WT R+ condition than in the other three control conditions representing the background rate of RT stop. In factorial terms, this means selecting the sites for which a wald-test on the coefficient of the interaction between the WT strain and R^+ condition reveals a significant positive effect on the outcome, the D-ratio.

14. Load filtered data in R. For each site (here shown for the first input row), create a table recapitulating the RT stop, coverage, and relevant design factors across <n> conditions.

```
rhoseq_data = read.table(combined.Dratio.filtered3, sep=''\t'')

dat=data.frame(''cov''=rhoseq_data[1,3:(n+2)], ''stop''=rhoseq_data[1,(n+3):((2*n)+2)],
''treatment''=factor(rep(c(R+, R-), n/2)), ''strain''=factor(rep(c(WT, dus, WT, dus)),
each=n/4))
```

Typically, the table should look like this assuming a balanced design with four conditions and two replicates:

Cov	Stop	Treatment	Strain	
[1]	122	25	R+	WT
[2]	145	8	R-	WT
[3]	117	5	R+	dus
[4]	102	6	R-	dus
[5]	99	19	R+	WT
[6]	133	3	R-	WT
[7]	87	4	R+	dus
[8]	112	0	R-	dus

15. Model the factors contribution in stopping RT in a generalized linear model of the binomial family with a logit link.

```
b_test = glm(cbind(dat$stop, dat$cov-dat$stop) ~ strain*treatment, family=binomial(link=
''logit''))
```

16. Extract the *p-value* associated with a positive effect of the strain:treatment interaction toward increased RT stop and save it in a vector.

```
pval_vector[1] = pnorm(summary(b_test)[[''coefficients'']][''strainWT:treatmentR+'',''z
value'']], lower.tail=F)
```

17. Correct *p-values* for multiple testing using with the Benjamini-Hochberg procedure (FDR).

```
padj_vector = p.adj(pval_vector, method=''FDR'')
```

18. Calculate the D-foldchange defined here as the ratio between the average D-ratio for the test conditions (WT R^+) and the average D-ratio for the control conditions (WT R^-, Δ4dus R^+, Δ4dus R^-).

```
Dratio_test_mean = rowMeans(dat[, Dratio_test_index])

Dratio_control_mean = rowMeans(dat[, Dratio_control_index])

Dfc = Dratio_test_mean / Dratio_control_mean
```

19. Select a list of putative D-sites according to p.adj < threshold (0.05 in this example) and average D-fold change > 4.

```
putative_D_sites = dat[which(Dfc > 4 & p.adj < 0.05),]
```

LIMITATIONS

Rho-seq relies on RT-stop resulting from rhodamine-labeled bases. However, we found that the sequence context surrounding modified sites affects rhodamine propensities for stopping the RT enzyme. For instance, a 150 nt spike-in sequence devoids of particular sequence structure and containing one dihydrouridine residue causes at most 20% RT stops after Rho-seq, while up to 80% was observed in the D-loop of some tRNAs (Finet et al., 2022). Such context-dependent variations are not unexpected given previous literature (Hauenschild et al., 2015), yet it limits out interpretation of within-sample comparisons of RT stop rate.

In theory Rho-seq can identify multiple modifications in virtually any organism. However, its application is restricted in practice by the requirement for control conditions in which the enzyme(s) responsible for the modification studied are deleted (Table 1). Depending of the modification, the enzyme might be unknown, or the studied organism might not be amenable to easy genetic manipulation, which is less of a problem with the increasing development of CRISPR technologies.

While Rho-seq allows the determination of modified nucleotide position at a single nucleotide resolution on the full transcriptome, it still has some blind spots. In particular, RT-stop events happening in close proximity to (less than 50 nucleotides away from) the RNA 3′-end cannot be observed because the smallest cDNA fragments are eliminated during library preparation. This issue can be mitigated by decreasing the stringency of the various washes (by increasing the beads:sample ratio), but there is a limit to the mitigation since it is essential that the washes remain sufficiently stringent to efficiently eliminate the extra adaptors and primers from the library.

TROUBLESHOOTING

Problem 1

The RNA concentration measured in step 2.q is too low (< 0.3 μg/μL).

Potential solution

The cause is probably either sample loss or degradation that could have occurred during the rhodamine labeling treatment. Skip directly to step 60 to run the samples on a bioanalyzer RNA pico chip (Figure 5B). If the RNA is degraded, remake solution from stock and clean surfaces before starting over.

Problem 2

Cy5 signal measured in the dot blot assay of step 3.b is saturated.

Potential solution

The exposition time is too high for the highly fluorescent rhodamine control drop. Decrease exposition time to <0.5 s to obtain images similar to Figure 5A.

Problem 3

RNA 6000 Pico bioanalyzer analysis in step 6.b reveals that the RNA is degraded after the R+/R- treatments.

Potential solution

The acidic treatment required for rhodamine labeling causes RNA degradation. It is therefore normal to observe a drop in RIN down to 6–7 after rhodamine labeling (Figure 5B). If RIN < 6, verify

that the pH of the reduction solution is not too low (like with pH < 4 in Figure S3A) and that the samples were not exposed to other sources of degradation such as an RNase contamination in buffers or tubes or an extended exposure to high temperature.

Problem 4

The bioanalyzer analysis in step 6.b reveals that 18s and 25s rRNA peaks are still visible (Figure 5B).

Potential solution

Ribodepletion was inefficient. Verify that the probes used for ribodepletion are compatible with the organism studied. If so, carefully repeating the ribodepletion procedure (steps 4 and 5) should get rid of most remaining ribosomal RNA.

Problem 5

The beads take too long to completely dry in step 8.i.

Potential solution

It is probable that some ethanol remained on the tube inner surface. If after 5 min the beads are not completely dry, spin them, remove remaining ethanol, and air-dry them 5 extra minutes on the magnetic rack.

Problem 6

The bioanalyzer analysis in step 15.b reveals discrete peaks of short DNA species.

Potential solution

Discrete peaks of short DNA species are usually primers/adaptor contamination. For instance, a discrete 60-nt peak is most likely caused by excess PCR primer. Repeat AMPure XP bead clean-up (steps 14.e–14.f) to get rid of all remaining adaptors/PCR primers.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Damien Hermand (damien.hermand@unamur.be).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The accession number for the data reported in this paper is GEO: GSE145685.

The codes generated during this study are publicly available on GitHub (www.github.com/cyaguesa/Rho-seq/).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101369.

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AUTHOR CONTRIBUTIONS

O.F. developed and optimized the experimental part of the protocol. C.Y.-S. developed the computational part of the protocol and wrote the manuscript with inputs from all authors. D.H. conceptualized and supervised the study. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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