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► To cite this version:

Ji Wang, Claire Toffano-Nioche, Florence Lorieux, Daniel Gautheret, Jean Lehmann. Accurate characterization of *Escherichia coli* tRNA modifications with a simple method of deep sequencing library preparation. *RNA Biology*, 2020, pp.1-14. 10.1080/15476286.2020.1790871 . hal-02898094

HAL Id: hal-02898094

<https://hal.science/hal-02898094>

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This article has been accepted for publication in RNA BIOLOGY, published by Taylor & Francis.

See the published version (later version) here:

- DOI : [10.1080/15476286.2020.1790871](https://doi.org/10.1080/15476286.2020.1790871)
- PUBMED : [32618488](https://pubmed.ncbi.nlm.nih.gov/32618488)

(the present version is a revised version for RNA biology, therefore before decision of acceptance by RNA BIOLOGY)

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3 **Accurate characterization of *Escherichia coli* tRNA modifications**
4 **with a simple method of deep sequencing library preparation**
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6

7 Ji Wang, Claire Toffano-Nioche, Florence Lorieux, Daniel Gautheret¹, Jean Lehmann¹

8 *Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud,*
9 *Campus Paris-Saclay, 91198 Gif-sur-Yvette, France*

10
11 ¹ corresponding author

12 daniel.gautheret@u-psud.fr; jean.lehmann@u-psud.fr

13
14 **ABSTRACT**

15
16 **In conventional RNA high-throughput sequencing, modified bases prevent a large fraction of tRNA transcripts to**
17 **be converted into cDNA libraries. Recent proposals aiming at resolving this issue take advantage of the**
18 **interference of base modifications with RT enzymes to detect and identify them by establishing signals from**
19 **aborted cDNA transcripts. Because some modifications, such as methyl groups, do almost not allow RT bypassing,**
20 **demethylation and highly processive RT enzymes have been used to overcome these obstacles. Working with**
21 ***Escherichia coli* as a model system, we show that with a conventional (albeit still engineered) RT enzyme and key**
22 **optimizations in library preparation, all RT-impairing modifications can be highlighted along the entire tRNA length**
23 **without demethylation procedure. This is achieved by combining deep-sequencing samples, which allows to**
24 **establish aborted transcription signal of higher accuracy and reproducibility, with the potential for differentiating**
25 **tiny differences in the state of modification of all cellular tRNAs. In addition, our protocol provides estimates of**
26 **the relative tRNA abundance.**

27
28
29 **INTRODUCTION**

30 During the past few years, several new experimental strategies have been developed to help characterize cellular
31 tRNAs from either cells or tissues, both in terms of modification state and relative frequency (Cozen et al. 2015; Zheng
32 et al. 2015; Hauenschild et al. 2015; Gogakos et al. 2017; Shigematsu et al. 2017; Schwartz et al. 2018). These
33 investigations came at the time of the realization that, although tRNA modifications are primarily ensuring an
34 accurate and efficient deciphering of mRNAs during translation (reviewed in Krutyholowa et al. 2019), a multitude of
35 other biological processes are directly or indirectly impacted by them (Novoa et al. 2012, Chan et al. 2018, Baldrige

36 et al. 2018). Furthermore, a large variety of diseases originate from an absence of tRNA modifications (Bohnsack and
37 Sloan 2017; Jonkhout et al. 2018, de Crécy-Lagard et al. 2019), while the modification state of tRNAs has also been
38 shown to relate to metabolism (Alexandrov et al., Richter et al. 2018; Ng et al. 2018; Pollo-Oliveira and de Crécy-
39 Lagard 2018). It is, therefore, highly desirable to develop straightforward methods for the characterization of all
40 tRNAs. The presence of modifications on tRNA constitutes a burden in traditional deep-sequencing experiments
41 because they prevent RT enzymes from generating full cDNA transcripts by blocking their progression along the RNA
42 strand. Earlier protocols of cDNA library preparation relied on RNA adapters ligated to both ends of the RNAs for PCR
43 amplification, implying that aborted cDNA transcripts could not be amplified. RT enzymes may still bypass
44 modifications at a rate depending on the nature and position of the chemical groups attached to nucleosides, which
45 would often lead to nucleotide misincorporation. This information has already been used to identify modified
46 residues in RNA-seq databases established from these earlier protocols (Ryvkin et al. 2013, Vandivier et al. 2019).
47 Since then, procedures to amplify aborted cDNA transcripts have been developed, which have allowed to establishing
48 signals at the location of RT-impending modifications, and thus acquiring quantitative information about the state of
49 modification (Hauenschild et al. 2015, Zheng et al. 2015, Clark et al. 2016). Currents methods are reviewed in Helm
50 and Motorin (2017) and Motorin and Helm (2019). Some modifications, such as methyl groups, do almost not allow
51 any RT bypass when they are situated on the Watson-Crick face of bases. In order to overcome this issue, Cozen et
52 al. (2015) and Zheng et al. (2015) combined demethylase treatments with an exceptionally processive RT enzyme to
53 generate full-length cDNA transcripts at higher yields. Alternate procedures were developed by Motorin, Helm and
54 coworkers, who established aborted transcription signals from fragmented RNA (Hauenschild et al. 2015). Although
55 it is still used in many RNA deep-sequencing protocols (Hauenschild et al. 2015; Gogakos et al. 2017), RNA
56 fragmentation generates a high level of noise in the analysis due to ambiguous gene mapping. This procedure was,
57 therefore, avoided in the present study.

58 Searching for a method that could highlight RT-impairing base modifications with minimal benchwork, we identified
59 a robust procedure that allows to generate signals from aborted cDNA transcripts without requiring demethylase
60 treatment and/or RNA fragmentation, and that we validated with a regular RT enzyme (Superscript III, Invitrogen).
61 Working with 3 biological replicates from *Escherichia coli*, a bacterial species the tRNA modifications of which are
62 well characterized, we first show that conventional treatments of total RNA samples (DNase, DNase and deacylation)
63 have no significant effect on these signals (we call them **ts**, termination signals). The number of QC-passed reads
64 obtained from single samples (10-15 million) is, however, too low to fully characterize all tRNAs. Next, we show that
65 a significant gain in accuracy of **ts** signals is obtained by combining independently sequenced samples. Because deep-
66 sequencing experiments require PCR amplification, tiny variations in the amount of initial sample translate into
67 noticeable differences in relative fractions of amplified cDNA transcripts, implying that **ts** signals fluctuate around
68 characteristic values. Our data show that these fluctuations reach amplitudes as high as 90%. Pooling reads from 3
69 experiments allows us to demonstrate a reduction in **ts** standard deviation by a factor > 2. Furthermore, by combining
70 all samples from 9 independent deep-sequencing experiments, totalizing about 60 million mapped reads, we show

71 that the coverage is high enough to fully characterize 43 out of the 48 different *E. coli* tRNAs, 5 weakly expressed
72 tRNAs being incompletely covered with **ts** signals. *E. coli* being an extensively studied bacteria, almost all **ts** signals
73 can be attributed to modifications listed in the *Modomics* database (Boccaletto et al. 2018). An innovative aspect of
74 our protocol based on the conserved CCA 3' terminal of all tRNAs was found essential to obtaining highly consistent
75 data. Because the ultimate A76 residue, that is required for aminoacylation, can be specifically removed by RNase T
76 in *E. coli* (Deutscher et al. 1984, 1985), a subpopulation of non-functional tRNAs may always occur in these cells
77 (Czech 2020 and our unpublished data). Conversely, an additional CCA may be added by the CCA-adding enzyme that
78 tags these tRNAs for degradation through the combined action of poly(A) polymerase and RNase R (Li et al. 2002,
79 Mohanty et al. 2012, Wellner et al. 2018). We found that the use of primers with TGG 3' overhang in the first PCR
80 step essentially only amplifies CCA 3'-ending RNA transcripts and drastically reduces background amplification. This
81 enables our protocol to generate signals of unprecedented cleanliness, although it is restricted to almost only tRNAs.
82 The universality of the tRNA CCA 3' end makes it, however, applicable to any organism of interest. Because all
83 transcribed cDNAs start at the 3' end of the tRNAs, a relative quantification of mature isoacceptors can be established
84 from the read coverage at that position.

85
86

87 **MATERIAL & METHODS**

88 **Preparation of samples**

89 To test the proposed method, three biological replicates (R1, R2 and R3) of total RNA from *E. coli* MG1655 cells grown
90 at 37°C in standard LB medium were sampled at an OD₆₀₀ of ~4.0. Total RNA was extracted with trizol/chloroform.
91 Glycogen was added and the solution was precipitated with ethanol. Each of these samples were prepared following
92 three different procedures (Fig. 1): Total RNA was either not treated (**a**), or treated with DNase (**b**), or treated with
93 DNase and deacylated (**c**) prior to 3' adapter ligation (Table 1). DNase treatment (with TurboDNase, Ambion) was
94 performed 4 hours at 37°C, followed by overnight incubation at 4°C. Adapter ligation (10°C overnight) was achieved
95 with a pre-adenylated RNA 3' adapter using a truncated T4 RNA ligase 2 (NEB). Reverse transcription was performed
96 with Superscript III (Invitrogen), 1h at 55°C. Chimeric RNA/cDNA products were directly loaded on gel for size
97 selection between 35 to 200 bp by electrophoresis to eliminate the excess of 3' adapters and select mid-size RNA
98 transcripts. Gel-extracted products were subjected to poly-A tailing by a TdT enzyme (NEB) 45 min at 37°C. A first
99 round of selective pre-amplification of the cDNA (10-18 cycles) was achieved using a Taq polymerase (Invitrogen)
100 with *Illumina* TruSeq sRNA RTP with TGG overhang and *Illumina* TruSeq sRNA 5' adapter primer with polyT(20). A
101 second amplification (5-15 cycles) with *Illumina* RP1 and *Illumina* TruSeq sRNA Index primer (RPIX) that added a
102 specific tag to each of the 9 samples provided the final libraries. *Illumina* deep sequencing (libraries multiplexing;
103 pair-end: 2x125bp PE, HiSeq High Output mode, V4 chemistry) was performed by an external contractor (Genewiz).
104 The detailed protocol of library preparation is available in supplemental File S1.

105

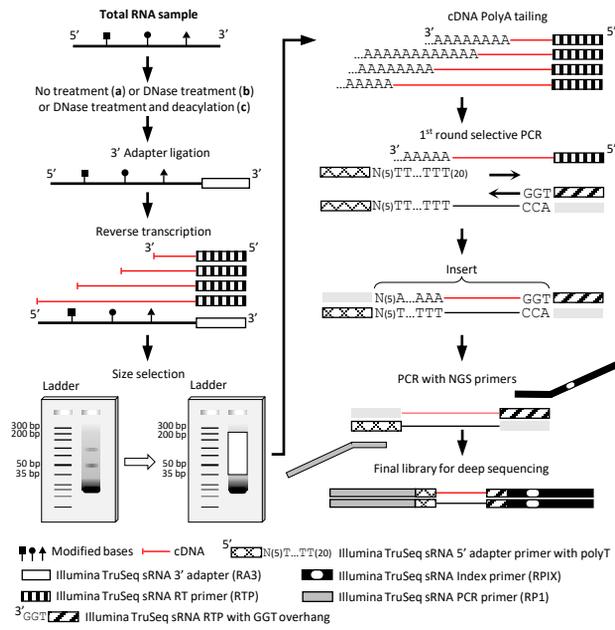


Figure 1. Workflow of library preparation. See text for explanations and Supplemental File S1 for detailed protocol. Note that the stretch of 5 random positions (N5) preceding the poly(T) tail of the 5' adapter primer is a requirement of Illumina deep-sequencing spot localization.

106 **Computational treatment of deep-sequencing data**

107 Processing of the deep-sequencing data required an optimization in order to extract the most relevant signal from
108 read coverage. We established a protocol (Fig. 2 and suppl. Fig. S1) that generated the sharpest termination signals
109 (see below). In brief, only reads R2 in pair-end sequencing (i.e. sequenced from the 3' end) were processed. Both
110 adapters were removed and poly-T tails corresponding to TdT-added poly A on cDNA were trimmed. Mapping on *E.*
111 *coli* K-12 (substr. MG1655) genome was achieved with Bowtie 2 (Langmead and Salzberg 2012) with the *--local*
112 option, that maximizes the stretch of correct matches at the end of a read. This option turned out to be essential
113 because bases other than A sometimes occur in the added poly-A that prevent its complete removal at the trimming
114 step. Finally, a CCA/TGG filter complementing the selective PCR step only kept mapped transcripts with CCA at the
115 3' end. The quality and distribution of read lengths at each step, performed with *FastQC* (v0.11.5, *Babraham*
116 *Bioinformatics*), are shown in supplemental Figure S1. After filtering, about 5 million to 9 million reads mapped onto
117 the *E. coli* genome (Table 2; suppl. Fig. S1), allowing to establishing tRNA genomic coverages.

118

119 **Visualization**

120 To connect **ts** signals with modified bases, all *E. coli* tRNA sequences from the *Modomics* database (Boccaletto et al.
121 2018) were aligned onto the *E. coli* genome with *blastn* (ncbi-blast-2.5.0+), where a .gff file specifying the position of
122 661 modifications (suppl. File S2) allowed their visualization on IGV (Robinson et al. 2017). A separate .txt file (suppl.
123 File S3) was used to generate plots connecting **ts** signals with tRNA sequences and modifications (Fig. 3b).
124 Modifications one-letter-code are from the *Modomics* database (supplemental Table S1).

125

126

127 **RESULTS**

128 **Analytical treatment of tRNA transcripts coverages**

129 Examination of tRNA coverages (Fig. 3a) revealed that they were highly specific and reproducible. The coverages
130 decrease from the terminal CCA^{3'}, with sudden jumps at locations of RT-impairing base modifications. Following
131 analytical procedures introduced by Helm, Motorin and coworkers (Hauenschild et al. 2015) and Pan and coworkers
132 (Zhang et al. 2015; Clark et al. 2016) these jumps were converted into termination signals (**ts**) highlighting the loss of
133 coverage (in %) from nucleotide i+1 to nucleotide i (Fig. 3b). A **ts** signal is defined only when coverage (i) \geq 100 reads
134 to ensure a minimal precision of the order of 1%. Furthermore, when negative values occur (reflecting very rare
135 instances of amplified internal fragments), **ts** is set to zero. Figure 3a illustrates the analysis with the R1b sample of
136 *lysQ*, the coverage of which corresponds to the average expression of 6 identical genes (*lysQ*, *lysT*, *lysV*, *lysW*, *lysY*,
137 *lysZ*). The average **ts** signal established from the R1b, R2b and R3b samples (Fig. 3b) allows to highlight three major
138 termination events associated with base modifications (see below for the analysis). Although the amplitude of these
139 signals fluctuates among samples, they all correlate well (Fig. 3c and Table 3): Pearson correlation coefficients (**r**) are
140 all above 0.92, with 22/36 coefficients higher than 0.98 (all associated *p* values are zero). Two significant observations
141 can be made from Table 3. First, the amplitude of **ts** signals does not depend on RNA treatment: the two samples

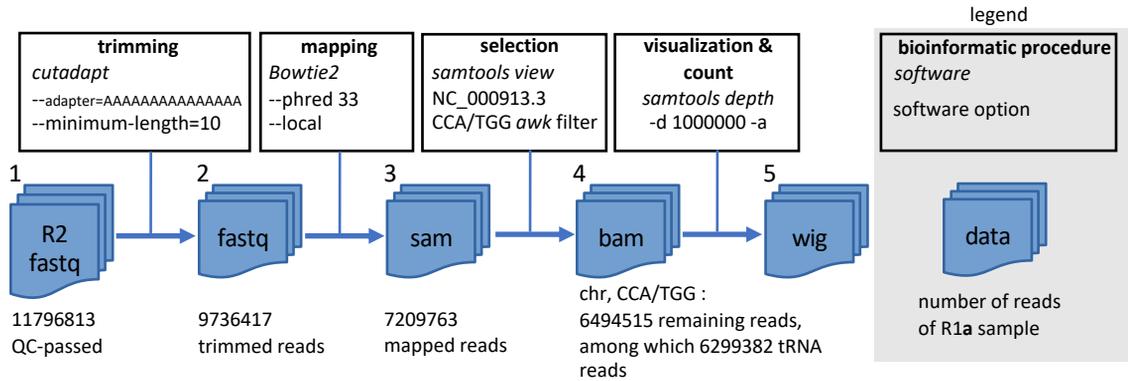


Figure 2. Computational workflow of deep-sequencing Illumina data processing (See Supplemental Figure S1 for details). Shown values are from the R1a sample (all samples provided similar results). Among half a dozen tested strategies, the established workflow provided the sharpest termination signals. From the initial QC-passed reads (1), trimmed reads (2) were obtained with cutadapt by identifying and trimming all TdT-added poly-A (i.e. poly-Ts on the tRNA gene sequence) with at least 15 nt. In a second step, a local mapping of the reads onto the *E. coli* genome with bowtie2 (Langmead and Salzberg 2012) was achieved, providing the mapped reads (3). The `-local` option maximizes the stretch of matching bases by removing mismatches at the end of the reads (softclipping). In a third step, mapped reads were filtered with a custom awk script to select only the reads ending with CCA/TGG (depending to the orientation of the tRNA on the genomic sequence), thus complementing the selection of mature tRNA achieved during library preparation. This operation provided the final mapping (4), from which a wig file (5) compiling the coverage of all expressed RNA genes was established with samtools depth using the `-d 1000000` and `-a` options.

Treatment	Biological replicate		
	R1	R2	R3
a. no treatment	R1a	R2a	R3a
b. DNase	R1b	R2b	R3b
c. DNase + deacylation	R1c	R2c	R3c

Table 1: The 9 total RNA samples analysed in this study.

Sample	Total reads mapped and CCA/TGG filtered	tRNA	pseudo tRNA	tmRNA
R1a	6,494,515	6,299,382 (97,0%)	45,775 (~1%)	1,191 (~0.01%)
R2a	5,150,196	5,026,263 (97,6%)	44,260 (~1%)	704 (~0.01%)
R3a	6,941,871	6,793,490 (97,9%)	67,948 (~1%)	426 (~0.01%)
R1b	7,229,758	7,056,586 (97,6%)	91,245 (~1%)	851 (~0.01%)
R2b	6,803,833	6,669,159 (98,0%)	45,793 (~1%)	2,336 (~0.01%)
R3b	7,551,225	7,385,017 (97,8%)	103,008 (~1%)	749 (~0.01%)
R1c	9,045,057	8,899,275 (98,4%)	54,894 (~1%)	1,074 (~0.01%)
R2c	7,457,325	7,301,761 (97,9%)	54,527 (~1%)	3,378 (~0.01%)
R3c	6,771,946	6,598,669 (97,4%)	82,914 (~1%)	1,515 (~0.01%)

Table 2: Total read numbers of samples. Reads of tRNA are from the following genes (identical copies are indicated in brackets): (*alaT, alaU, alaV*), (*alaX, alaW*), (*argQ, argV, argY, argZ*), (*argU*), (*argW*), (*argX*), (*asnT, asnU, asnV, asnW*), (*aspT, aspU, aspV*), (*cysT*), (*glnU, glnW*), (*glnV, glnX*), (*gltT, gltU, gltV, gltW*), (*glyT*), (*glyU*), (*glyV, glyW, glyX, glyY*), (*hisR*), (*ileT, ileU, ileV*), (*ileX*), (*ileY*), (*leuP*), (*leuQ, leuT, leuV*), (*leuU*), (*leuW*), (*leuX*), (*leuZ*), (*lysQ, lysT, lysV, lysW, lysY, lysZ*), (*metT, metU*), (*metV, metW, metZ*), (*metY*), (*pheU, pheV*), (*proK*), (*proL*), (*proM*), (*selC*), (*serT*), (*serU*), (*serV*), (*serW, serX*), (*thrT*), (*thrU*), (*thrV*), (*thrW*), (*trpT*), (*tyrT, tyrV*), (*tyrU*), (*valT, valU, valX, valY, valZ*), (*valV*), (*valW*). Reads of pseudo tRNA are from the following genes: (*pauD*), (*pawZ*), (*ptwF*). Reads of tmRNA are from the following gene: (*ssrA*).

	R1a	R2a	R3a	R1b	R2b	R3b	R1c	R2c	R3c
R1a	1.0000	0.9643	0.9584	0.9463	0.9565	0.9623	0.9611	0.9896	0.9686
R2a		1.0000	0.9965	0.9892	0.9926	0.9930	0.9909	0.9424	0.9861
R3a			1.0000	0.9937	0.9937	0.9926	0.9935	0.9377	0.9887
R1b				1.0000	0.9928	0.9871	0.9923	0.9272	0.9876
R2b					1.0000	0.9890	0.9968	0.9398	0.9844
R3b						1.0000	0.9889	0.9422	0.9853
R1c							1.0000	0.9455	0.9892
R2c								1.0000	0.9566
R3c									1.0000

Table 3: Pearson correlation coefficients (*r*) between samples *ts* values. Correlation coefficients established from *ts* signals of tRNA, tmRNA and pseudo tRNA only when these signals are defined in all 9 samples (*N* = 3146 per sample); see Table 2 for the list of genes. Identical transcript copies (e.g. *metT, metU*) are included only once in the analysis. Dark green: $0.92 \leq r < 0.95$; medium green: $0.95 \leq r < 0.98$; light green: $r \geq 0.98$. All associated *p*-values are 0.

<i>ts</i> threshold	A. 1 sample (a, b or c)	B. Avg., 2 samples (all comb.)	C. Avg., 3 samples (a, b, c)	ratio A/C
$ts_{R1+R2+R3} > 0$	0.719 (N = 66781)	0.456 (N = 68775)	0.324 (N = 2538)	2.22
$ts_{R1+R2+R3} > 1$	1.952 (N = 23239)	1.278 (N = 23070)	0.920 (N = 839)	2.12
$ts_{R1+R2+R3} > 10$	5.230 (N = 6977)	3.467 (N = 6863)	2.520 (N = 249)	2.08
$ts_{R1+R2+R3} > 20$	6.249 (N = 5204)	4.177 (N = 5172)	3.033 (N = 188)	2.06

Table 4: Comparison between biological replicates R1, R2 and R3: average *ts* standard deviation (%) as a function of the number of samples (a, b and/or c), established for four *ts* threshold. $ts_{R1+R2+R3} > x$ implies that a particular standard deviation is computed only if the sum of *ts* signals of the three replicates at a given position is $> x$. About 2,500 *ts* values are simultaneously determined in all three replicates for a considered set of samples (the total number of nucleotide positions, including all tRNAs and three tRNA pseudogenes, is 3,916). Standard deviations are established for all possible combinations of samples. With one sample (A), there are $3^3 = 27$ combinations. With two samples (B), there are also $3^3 = 27$ combinations (ex: avg. (R1a, R1c); avg. (R2a, R2b); avg. (R3b, R3c)), whereas only one possibility occurs with three samples (C).

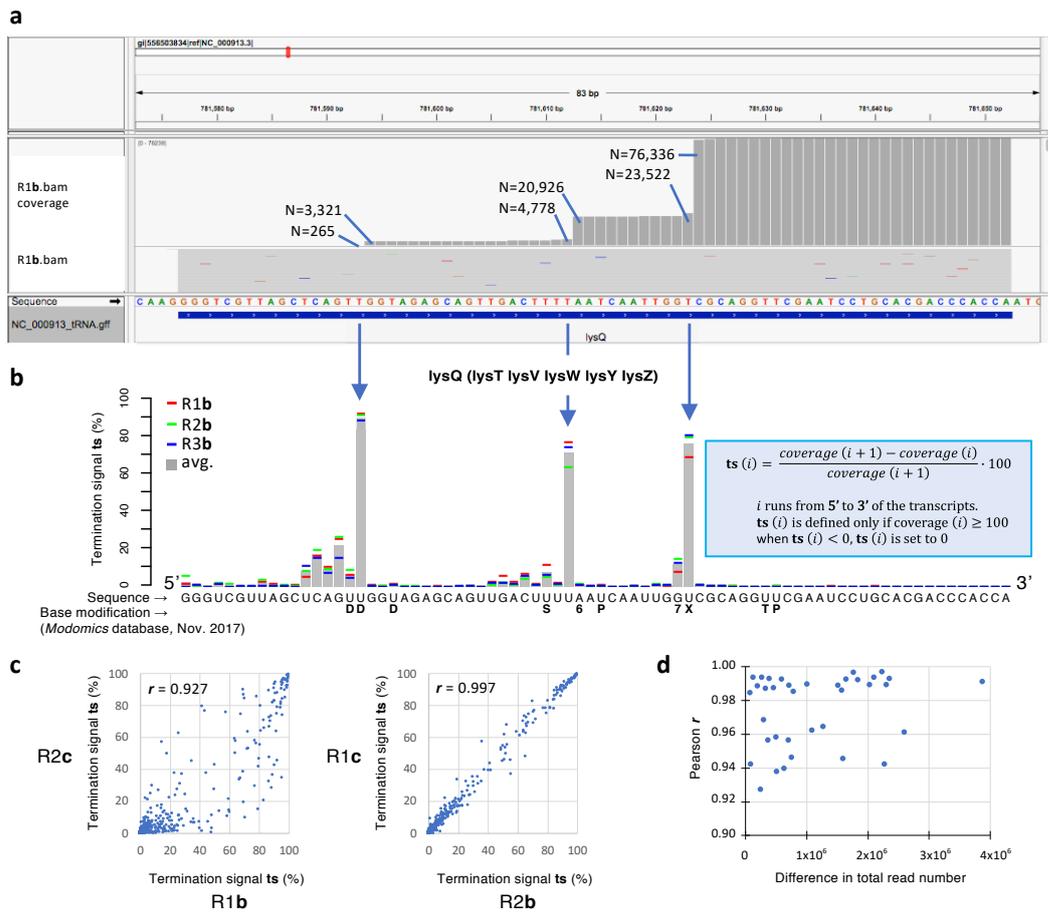


Figure 3. Overview of the analysis of deep-sequenced CCA3'-amplified RNA transcripts. a) Snapshot of an IGV window (Thorvaldsdóttir et al. 2013) centered on the *lysQ* tRNA gene of *E. coli* MG1655 genome, showing the coverage established from the R1b sample. This gene is oriented 5'-3' from left to right. Interruption of reverse transcription events (from 3' to 5') generate coverage jumps. b) Termination signals (*ts*, defined in the box) of *lysQ* tRNA, established from the coverage of R1b, R2b and R3b samples. Because the *lysQ* sequence is identical to that of *lysT*, *lysV*, *lysW*, *lysY* and *lysZ*, reads are evenly distributed among these 6 genes. Base modifications symbols indicated below the sequence are from the Modomics database (Boccaletto et al. 2018) (Supplemental Table S1). c) Ts-ts Plots illustrating the lowest (left) and the highest (right) obtained Pearson correlation coefficients (*r*) between samples (see Table 3); *n* = 3146 in both plots. d) Pearson correlation coefficients as a function of the difference in total tRNA read number between samples.

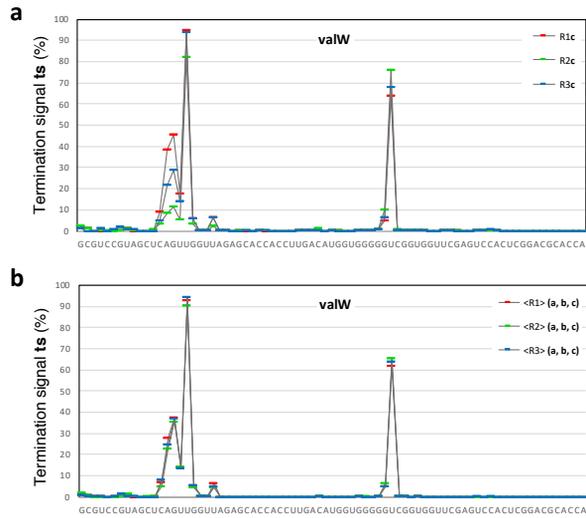


Figure 4. Gain in precision obtained in the ts analysis when ts signals from 3 samples are averaged, as compared to signals obtained from single samples (illustration with valW tRNA). a) Comparison of ts values between three biological replicates (R1, R2 and R3) with a single sample (c treatment). b) Same comparison as in a, but with ts values of each biological replicate averaged over three samples (a, b and c). Because RNA sample treatment (a, b, c) were found not to noticeably alter ts signals (see Table 3 and text), they are considered as equivalent. For a global quantitative analysis, see Table 4.

142 related by the lowest r (0.927) are R1**b** and R2**c** (Fig. 3c, left), while the highest r (0.997) is observed between the R2**b**
143 and R1**c** samples (Fig. 3c, right). It suggests that the difference in **ts** signal in between samples is essentially related
144 to PCR amplification. Remarkably, these signals are not affected by the total read number of a sample (Fig. 3d), which
145 constitutes an unexpected robustness of the method. It can thus be concluded that **b** and **c** treatments do not have
146 noticeable effects on the **ts** analysis, and, within the range of values obtained in the present study (Table 2), **ts** signals
147 are not affected by the total number of reads provided by a deep sequencing experiment. Because these signals
148 display fluctuations that can be higher than 90%, single deep-sequencing experiments cannot accurately determine
149 their characteristic values (Fig. 4a), a quantitative aspect that has not been addressed in earlier analyses based on
150 aborted cDNA signals (e.g. Hauenschild et al. 2015, Zhang et al. 2015). Since **ts** signals were found not to be affected
151 by total RNA treatments, we established the average **ts** standard deviation by combining **a**, **b** and **c** samples in each
152 replicate R1, R2 and R3, and found an increase in the accuracy by a factor > 2 when three samples were combined
153 (Fig. 4b; Table 4), the average standard deviation dropping from 6 to 3 % for **ts** signals equal or higher than ~ 7 % (i.e.
154 $ts_{R1+R2+R3} > 20$). The decrease of the average standard deviation with the number of samples being approximately
155 logarithmic, our data suggest that 7 to 8 independent sequencings provide signals ~ 10 times more accurate than
156 single ones (supplemental Fig. S2). Furthermore, when samples are separately analyzed, from 11 to 23 tRNAs
157 sequences lack full 5' **ts** characterization due to low coverage at the 5' end. By combining the coverages of the 9
158 independently sequenced samples of the present study (Table 2), only about 3% of the combined tRNA sequence
159 length could not be characterized (magenta lines in Figure 5; see supplemental File S4). Combining independent
160 samples has, therefore, a double benefit: it significantly increases the precision in the value of **ts** signals, required to
161 assess the level of modification of residues, and allows to establishing a complete (or near complete) **ts** analysis of
162 all tRNA sequences.

163

164 **Termination signal analysis of *E. coli* tRNA transcripts**

165 The **ts** analysis generates signals similar to the arrest rate (Hauenschild et al. 2015) and modification index (Zhang et
166 al. 2015; Clark et al. 2016). It provides a set of characteristic signals reflecting the modification state of a tRNA. Some
167 modifications, such as **P** and **T**, can only be revealed by chemical treatments that make them interfering with RT
168 enzymes (Motorin et al. 2007, Zhang et al. 2019). These were not investigated in the present study. To facilitate a
169 rapid assessment of all tRNAs, four types of modifications are highlighted in Figure 5 (see supplemental Table S1 for
170 one letter code). Green dots indicate the **T** and **P** modifications of the T-loop, for which no interference with the RT
171 enzyme is noticeable. Blue dots highlight the **D** modification, with which strong **ts** signals are observed when three
172 or more of such residues are present (see below). Orange dots signal modifications for which strong **ts** signals are
173 observed only when two or more of such modifications are in close vicinity (**V**, **%**, **{**, **6**, **Q**, **c**, *****, **\$**, **S**, **B**, **M** and **+**) while
174 red dots highlight modifications that are always interfering strongly with the RT enzyme (**X**, **K**, *****, **}**, **)** and **E**).
175 Modifications that do essentially not affect the path of the RT enzyme (such as **4** and **#**) are left unhighlighted.

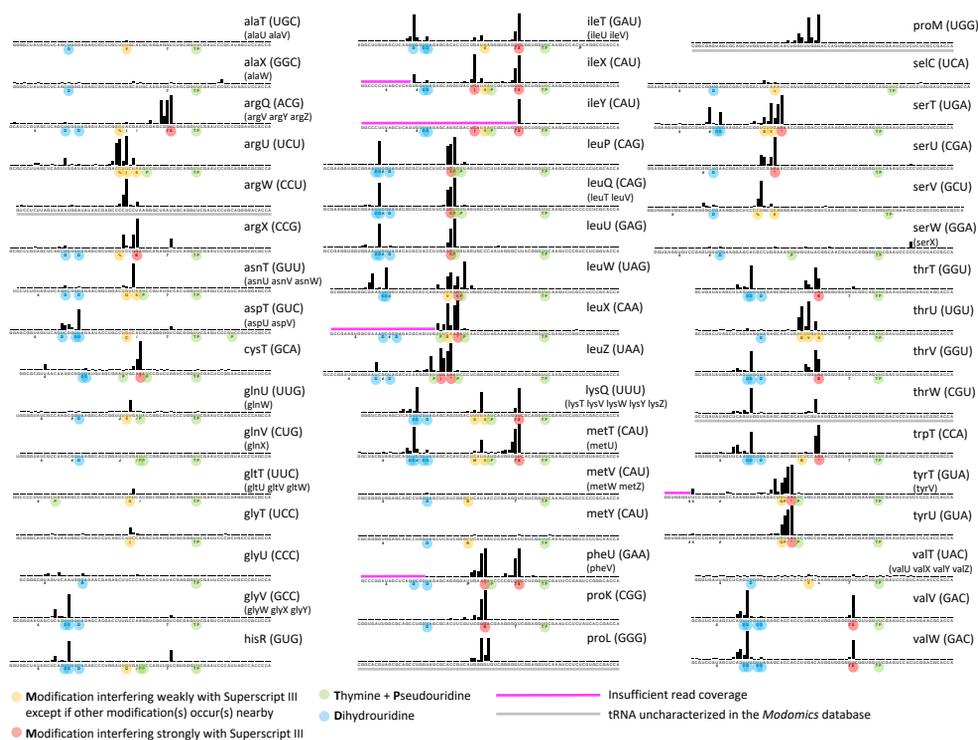


Figure 5. Atlas of ts signals of all *E. coli* tRNAs, with base modifications reported from Modomics (as of Nov. 2017), with one correction: only one D modification could be attributed to serW (serX). Modomics-uncharacterized tRNAs are underlined with gray lines. Identical tRNA genes are represented only once (copies are listed in brackets). Ts signals are established from the combined coverage of all 9 samples of the present work (Table 2). Magenta lines highlight 5' portions of tRNAs for which the total coverage was too low to establish ts signals. Plots with individual ts values for all 9 samples are available in Supplemental File S4.

176 When modified residues are close to each other, non-linear **ts** amplifications are observed, a situation that can be
177 attributed to the combined burden that these modifications impose to the core of the RT enzyme. It is thus often not
178 possible to relate specific **ts** signals with a given modification, although the *pattern* of these signals is most often
179 altered if a modification is missing. Also, when stretches of U residues occur on the 3' side of modified bases, the
180 position of **ts** signal(s) resulting from the interference with the RT enzyme is shifted to the 3' side due to poly-T
181 computational trimming on DNA reads (see Section "Computational treatment of deep-sequencing data" and
182 supplemental Fig. S3). This effect is highlighted in figures with blue arrows.

183 Following the path of the RT enzyme, **ts** signals are analyzed below from the 3' end to 5' end (Fig. 6a), crossing four
184 regions highlighted in green (from the 3' end to the 5' side of the T-arm), salmon (variable loop), sand (anticodon
185 stem-loop) and blue (from the D-loop to the 5' end).

186 **Region of the T-arm**

187 The length encompassing the 3' end of the tRNA to the 5' side of the T-arm being highly conserved (it comprises 28
188 nucleotides in *E. coli*, tRNA^{seI} being the only exception with 27 nts), we performed the **ts** analysis at once by combining
189 signals of all tRNAs (Fig. 6b). Only **T** and **P** modifications are present, which do not constitute an obstacle to the RT
190 enzyme (Motorin *et al.* 2007). From the 3' end, the first region presenting a significant (albeit very low) **ts** signal starts
191 after the entry of the T-arm (pos. 65; red arrow). It appears to be related to the RT enzyme encountering the densely
192 folded region of the tRNA, as suggested by a correlation between the proportion of (G+C) residues in the T-arm and
193 the summed **ts** signals along this segment (Fig. 6b, right inset). The line chart of Figure 6b reveals that the profile of
194 the average signal is highly reproducible: essentially only the amplitude varies among the 9 samples, an effect that
195 we attribute to PCR amplification. The **ts** signals of pos. 54 and 55 (**T** and **P**) combine on pos. 55 (blue arrow on Fig.
196 6b) as a result of poly-A computational trimming (supplemental Figure S3). The variability of the **ts** signal for individual
197 tRNAs (Fig. 6b, left inset) reveals that it is highly sequence dependent, even though the amplitude of this variability
198 is small.

199

200 **Region of the variable loop**

201 Only two modified bases are present at the start of the variable loop in some *E. coli* tRNA: 3-(3-amino-3-
202 carboxypropyl) uridine (**X**) and 7-methylguanosine (**7**). **X** is analog to N1-Methyl-3-(3-amino-3-carboxypropyl)-
203 pseudouridine, known to allow only minimal bypass by RT enzymes, while **7** was demonstrated not to detectably
204 affect reverse transcription (Motorin *et al.* 2007). According to the *Modomics* database, the **7** modification is present
205 in position 46 in slightly more than 50% of all *E. coli* tRNAs, and only when **7** is present does **X** occur in position 47 for
206 half of these tRNAs. Our data show that a strong **ts** signal is always present when **X** is listed in *Modomics* (Fig. 6c).
207 Furthermore, additional "trailing" **ts** signals occur on the 5' side of the major **ts** signal when it is higher than ~80%
208 (Fig. 6d). Two important deductions can be made from our results: Figure 6c shows that the **ts** signal associated with
209 **X** cannot unambiguously separate **7U** from **7X**-modified tRNAs, suggesting that all tRNAs with **7U** in positions 46-47
210 are converted to **7X** to some extent although they may not be listed as such in *Modomics* (prominent cases are *argX*

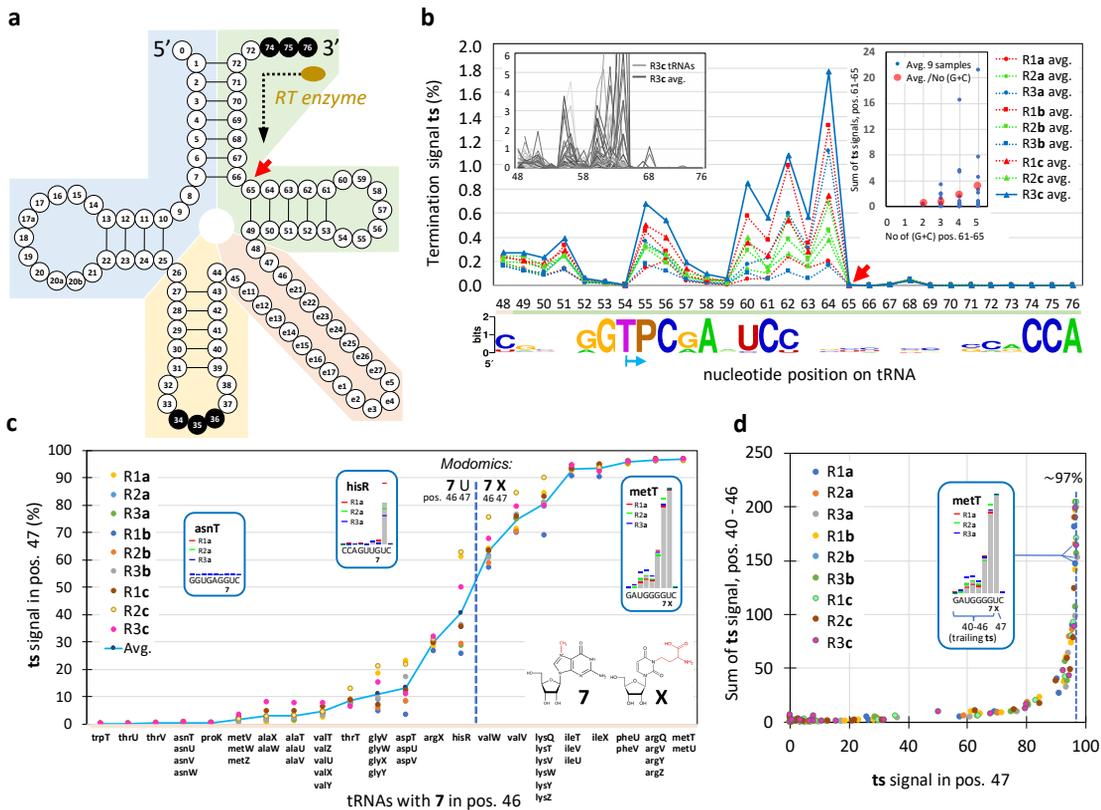


Figure 6. a) Standard tRNA nucleotide numbering according to the tRNA gene database curated by experts website (Abe et al., 2014). The colored regions correspond to the four successively analyzed tRNA segments, from 3' to 5': green, red, sand and blue. b) Ts analysis from pos. 76 to pos. 48 (green segment), established from an alignment of all tRNA transcripts. The sample with the highest values (R3c) is highlighted with a thick line. The left inset illustrates individual tRNA values (and the average) of the R3c sample. The right inset shows the sum of the ts signal as a function of the number of (G+C) residues in positions 61 to 65. Each blue dot corresponds to a tRNA; values averaged over all 9 samples (see Table 1). Red dots correspond to values averaged over all tRNAs. The base conservation logo reported at the bottom is based on all *E. coli* tRNA listed in Modomics. Because T and P modifications correspond to a stretch of two As on the cDNA, the ts signal of the two residues essentially combine into a single signal on the P residue (blue arrow) (see Material and Methods). c) Ts analysis of the variable loop (red segment). Ts signal in pos. 47 for all tRNAs bearing the 7 modification in pos. 46, according to Modomics. For tRNAs with multiple gene copies (e.g. asnT, asnU, asnV, asnW), only one set of values is indicated. tRNA genes are ranked from left to right following an increasing average value of the ts signal. The vertical dashed line splits tRNAs into those with U (left) from those with X in pos. 47 according to Modomics (as of Nov. 2017). Insets illustrate three ts signals. d) Sum of ts values from pos. 40 to 46 (trailing ts signals) as a function of the ts value in pos. 47. Note that according to the Modomics database, all *E. coli* tRNAs with 7 and X modifications do not have any e residue in the variable loop, implying that the sequences analyzed in c and d follow standard numbering (panel a).

211 and *hisR*). This observation corroborates biochemical analyses performed by Meyer et al. (2019), who showed that
212 the modifications of these two residues are interdependent. Furthermore, the value of the **ts** signal in position 47
213 converges towards ~97% when the overall signal reaches the highest values (Fig. 6d). Assuming that the fraction of
214 **X**-modified tRNAs reaches 100% in that case, the bypass rate of this modification by the RT enzyme is of about 3%,
215 the y-axis of Fig. 6c thus representing roughly the percentage at which U47 is converted into X on the tRNAs. A
216 straightforward interpretation of trailing **ts** signals is that RT enzymes bypassing the **X** modification drop off one or a
217 few nucleotides downstream as a result of the perturbation caused by the modification embedded in the chimeric
218 cDNA/RNA duplex.

219 **Region of the anticodon loop**

220 The anticodon loop is by far the region with the most diverse types of modifications (Boccaletto et al. 2018). Figure
221 7 and supplemental Figure S4 highlight **ts** signals generated by some of the most frequent *E. coli* modifications.

222 **N6-threonylcarbamoyladenosine (6)**. This large modified base appears to constitute a moderate obstacle to the RT
223 enzyme (Fig. 7a, b, d, e, h). Unexpectedly, a major **ts** signal is not present at the location of the modification, but one
224 base downstream the path of the RT enzyme (small red arrow), suggesting that it generates an impediment only after
225 being incorporated into the cDNA/RNA duplex. An examination of the read processing revealed that the observed **ts**
226 pattern (Fig. 7a) is partly the result of incomplete 5' poly-A trimming due to the incorporation of other bases (often
227 a C) in the poly-A, followed by local mapping (supplemental Fig. S3). Thus, the pattern of **ts** signals associated with a
228 modification may not always be interpreted as a direct consequence of the interaction between the modification and
229 the RT enzyme. *Interference of 6 with other modifications*: **6**, that is only found in pos. 37, is often accompanied by
230 other modifications in pos. 34 (**Q**, **S**) or in pos. 32 (**%**). Figure 7b, 7e and 7h show that whenever this occurs, the
231 combined perturbations generate a **ts** response that is stronger than a linear combination of individual **ts** signals (see
232 below).

233 **Queuosine (Q)**. The effect of this large modification on RT processivity was not assessed in the seminal work of
234 Motorin et al. (2007). Surprisingly, it does not generate a strong **ts** signal (Fig. 7c), suggesting that the RT enzyme can
235 easily cross this residue if it is not in the vicinity of other modifications (Fig. 7b).

236 **5-methylaminomethyl-2-thiouridine (S)**. This modification generates a small **ts** signal (Fig. 7f) similar to **Q** (Fig. 7c).
237 When combined with **6**, the resulting **ts** signal (Fig. 7e) is similar to that of the **6-Q** combination (Fig. 7b).

238 **2-thiocytidine (%)**. The nearly isosteric C2-O → C2-S modification of C does not generate any **ts** signal when it is in
239 an unperturbed environment such as the one shown in Figure 7i. In that case, the closest modified neighbor is an
240 inosine (**I**), which is known not to interfere with the RT enzyme, although it leads to the incorporation of C and U
241 (Motorin et al, 2007). However, in the context of a **6** modification in pos. 37 (Fig. 7h), **%** generates an unexpectedly
242 high **ts** signal, suggesting that additional modification(s) unlisted in *Modomics* could be present on the **%** residue in
243 that case.

244 **2-methyladenosine (/)**. This modification was found to only generate RT pause at low dNTP concentration (Motorin
245 et al. 2007). Representative examples (Fig. 7c, f, i) show that it is indeed associated with almost no **ts** signal.

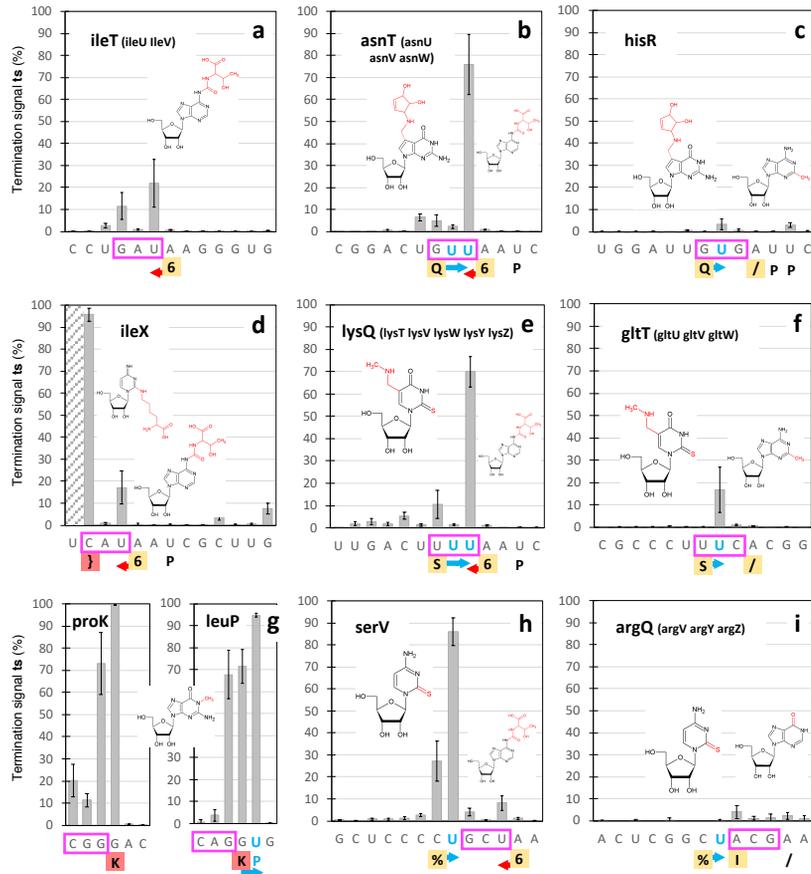


Figure 7. Ts analysis of the anticodon loop. Plots of the average values and standard deviations established from all 9 samples (Table 1), highlighting ts signatures of some typical *E. coli* combinations of modifications (panels a to i) and their ts error that occur when only a single sample is considered. Anticodons are boxed in magenta. Known modifications are reported from the Modomics database below the sequences (5'-3'). Highlighted modifications (in orange and red) are illustrated above in the same order (cartoons are from the Modomics website). A blue arrow indicates a shift of a ts signal associated with a modification as a result of the presence of U residue(s) on its 3' side (highlighted in blue), an artifact caused by poly-T computational trimming (see Material and Methods). Red arrows highlight the shift of the ts signal to the 5' direction observed with the 6 modification. The ts signal could not be established on the 5' side of the } modification for IleX (panel d, dashed lines) in all samples. Although Inosine (I) is associated with a low ts signal (panel i), an examination of the read coverage shows that it leads to incorporation of 86%C and 14%U on the cDNA, a typical signal of the presence of this modification (Schwartz and Motorin, 2017).

246 **2-lysidine (J)**. Only one *E. coli* tRNA bears this large modification, that is associated with a strong **ts** signal (Fig. 7d).
247 **1-methylguanosine (K)**. This small modification is known to constitute a major obstacle for the RT enzyme (Motorin
248 et al. 2007, Zheng et al. 2015). This can clearly be seen in our data: with *proK* tRNA (Fig. 7g, left), the average **ts** signal
249 at the position of the modification is as high as 99.65 ± 0.08 %, corresponding to a coverage drop from 283,925 to
250 864 reads with the **AD** sample. Assuming a rate of modification of 100% in that case implies a bypass rate of the order
251 of 0.35%. As a result of computational trimming, the major **ts** signal is shifted when a pseudouridine (**P**) is present on
252 its 3' side (Fig. 7g, right).

253 **2-methylthio-N6-isopentenyladenosine (*)**. This base has a complex pattern of modifications that is expected to
254 strongly impair the RT enzyme. It consistently generates a high **ts** signal (supplemental Fig. S4a). All related tRNAs
255 only have **7**, **T** and **P** modifications upstream the path of the RT enzyme, which do not affect its processivity. The only
256 exception is *pheU* (*pheV*), that bears the dual **7X** modifications in the variable loop. Although this early roadblock
257 generates a strong **ts** signal in these tRNAs, our data show that the **ts** signal at the * position is identical to that of
258 other tRNAs (supplemental Fig. S4b), demonstrating that **ts** signals generated by distant modifications along
259 transcripts may be considered as independent.

260 **Uridine 5-oxyacetic acid (V)**. In a way similar to the % modification, **V** essentially does not generate any **ts** signal
261 when occurring in a sequence context without perturbing modifications (supplemental Fig. S4c, d). When other
262 nearby modifications are present, such as in the case of *LeuW*, the effect of this base is difficult to assess because
263 stretches of U are present (supplemental Fig. S4e).

264 **2'-O-methylcytidine (B)**. This modification is known to generate RT pauses at low dNTP concentration (Motorin et al.
265 2007). In a sequence context without any other modified base, **B** does only generate a faint **ts** signal similar to **V**
266 (supplemental Fig. S4f). With a * modification 6 bases upstream the path of the RT enzyme, the associated **ts** signal
267 is slightly more pronounced (supplemental Fig. S4g), and with an additional **V** residue in between **B** and *, a strong
268 and complex **ts** pattern is observed (supplemental Fig. S4h). This last example illustrates the non-linear response of
269 the **ts** signal in the presence of three modifications, two of which do almost not generate any signal when found
270 isolated (supplemental Fig. S4c, f).

271 **Region of the D-loop**

272 **Dihydrouridine (D)**. With 152 reported instances, **D** is the most frequent tRNA modification found in *E. coli* *MG1655*
273 tRNAs according to the *Modomics* database. Because the D-loop is located near the 5' end, there is often not much
274 exploitable signal in each sample due to 3' upstream termination events caused by RT-blocking modifications (Fig.
275 3a). However, with the coverage provided by 9 deep-sequencing samples, accurate **ts** signals can be established up
276 to the 5' end for all but 5 weakly expressed tRNAs (Fig. 5). **D** is known to only create RT pauses (Motorin et al. 2007).
277 The presence of major **ts** signals associated with this modification therefore came as a surprise. It turns out that these
278 signals are highly dependent on the number of dihydrouridine(s) known to occur on the D-loop, a phenomenon
279 already noticed by Clark et al. (2016), although our quantitative analysis leads to a different conclusion with regard
280 to the effect. Considering the sum of **ts** signals in 11-nt windows centered on each dihydrouridine, a near clear-cut

281 threshold separates loops with one and two from those with three and four dihydrouridines when the window with
282 highest **ts** sum is considered (Fig. 8a,b). No clear sequence context allows to predict the position of the major **ts**
283 signal, which can be associated either with a doublet of dihydrouridines (*glyV*) or with one dihydrouridine (*ileT*).
284 Furthermore, it can occur 'early' (*aspT*) or 'late' (*ileT*) along the path of the RT enzyme (Fig. 5). Clark et al. (2016)
285 found a strong signal associated with a doublet of dihydrouridine in human tRNA^{asn} (GUU), and concluded that a
286 doublet sufficiently destabilizes the RT enzyme to lead to transcription abortion. Our analysis suggests instead that
287 all three U residues present on the D-loop of this tRNA are likely modified. Similarly, our analysis predicts the presence
288 of an unknown **D** modification on the D-loop of *leuW*, where only two dihydrouridines are listed in the *Modomics*
289 database. The **ts** signal analysis (Fig. 8b) and a sequence context that is identical to that of five other leucine tRNAs
290 where an analogous uridine is modified (Fig. 8d) are indeed both consistent with the presence of a third
291 dihydrouridine. With a high **ts** signal and only two possible **D** modifications on the loop, the case of *serT* is less clear
292 (Fig. 8d). Examination of the *Modomics* database reveals that whenever a uridine is present in the D-loop, it is
293 essentially modified into **D**, with two exceptions, *glyT* and *argU* (Fig. 8c). Our predictive analysis (Fig. 8b) suggests
294 that at least two among the four uridines present in the D-loop of *glyT* are indeed not modified (Fig. 8d), supporting
295 the possibility of this exception (the **ts** signal associated with *argU* cannot be unambiguously interpreted).

296

297 **Quantification of -CCA ending tRNA transcripts**

298 Since amplified 3' tRNA segments are always long enough to be uniquely mapped onto the genome (except for
299 identical tRNA genes), a relative frequency of tRNA isoacceptors can be established by reporting the number of reads
300 at the position of the 3' end (Fig. 9a; see also Fig. 3a). It is, however, important to keep in mind the non-linear nature
301 of PCR amplification, that changes RNA transcripts true relative frequencies. Figure 9a shows that the observed
302 frequencies are comparable among all three biological replicates, and that sample treatment (**a**, **b** or **c**) only has a
303 marginal effect, except for isoacceptors for which the amino acyl linkage is particularly resistant (*ile*, *val*) or prone to
304 hydrolysis (*ser*) (Matthaei *et al.* 1966). In the first case, deacylation increases these frequencies, while isoacceptors
305 that are already substantially deacylated without any treatment become proportionally less represented (*ser*). Our
306 data thus show that deacylation still occurs with **a** and **b** samples, that allowed the ligation of the RNA adapter. The
307 mild pH of the RNA adapter ligation step (pH 7.5; overnight incubation at 10°C) is likely responsible for this
308 phenomenon. Deacylated samples (**c**) provide a picture of the population of all tRNA isoacceptors regardless of their
309 aminoacylation state. Figure 9b shows that their relative frequency mainly follows that of their corresponding codons
310 in the *E. coli* genome, a phenomenon that is expected based on tRNA gene copy number (Fig. 9c) (Higgs and Ran
311 2009; Du *et al.* 2017). Transcripts of a group of isoacceptors (*gln*, *asp*, *ser*, *glt*, *val* *gly* and *ala*), however, appear
312 significantly underrepresented, a feature that was not observed in a study in which quantification was achieved by
313 probe hybridization on gel (Dong *et al.* 1996; supplemental Fig. S5). While we could not find a clear explanation for
314 this result, it is possibly related to the -CCA 3' end requirement of our protocol. The 3' terminal adenosine can be
315 specifically removed by RNase T in *E. coli* (Deutscher *et al.* 1984, 1985), in which case these tRNAs would not be

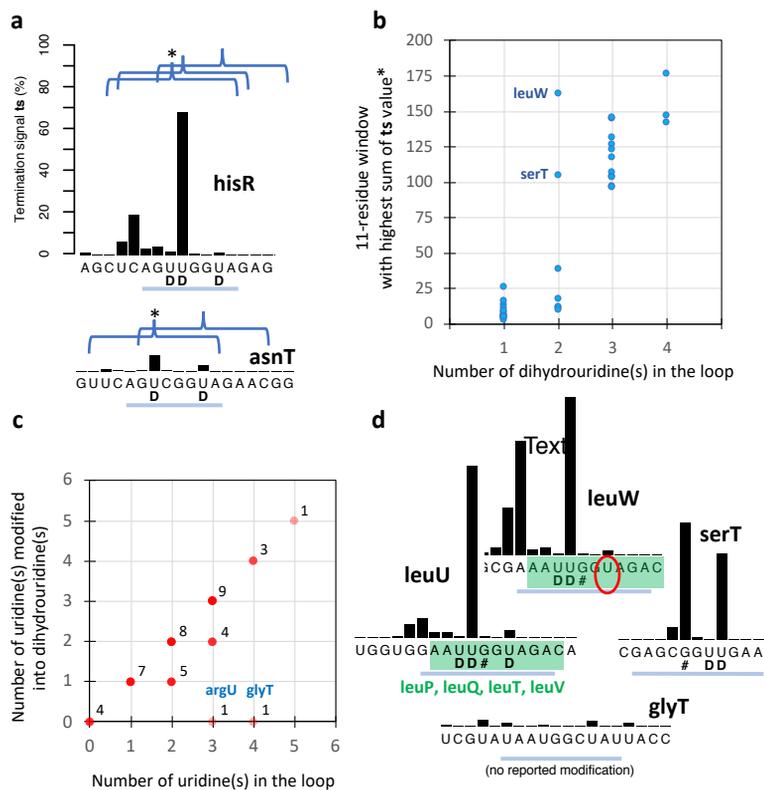


Figure 8. Ts analysis of the D-loop. a) Illustration of the effect of the presence of ≥ 3 (hisR, on top) and < 3 (asnT, bottom) dihydrouridine modifications. The curly brackets encompassing a window of 11 nts are centered on every reported dihydrouridine modification (this size allows to include most of the associated ts signal). b) Plot of the sum of ts signals in the window with the highest signal (*) as a function of the number of reported dihydrouridine modifications. c) Plots of the number of uridine(s) modified into dihydrouridine(s) in the D-loop according to the Modomics database (nov. 2017). In two cases, with 3 and 4 uridines (argU and glyT), no such modification has been reported. d) Ts signal and sequence similarity (in green) between leuU, leuP, leuQ, leuT, leuV, for which three dihydrouridines have been reported, and leuW, for which the ts analysis (in b) and sequence similarity predict the presence of a third unlisted dihydrouridine (circled). The low ts signal of glyT is consistent with the absence of at least two dihydrouridine modifications among the four uridines of the loop. Ts values are established from the combined coverage of all 9 samples (Table 1). D-loop segments are highlighted with a blue line.

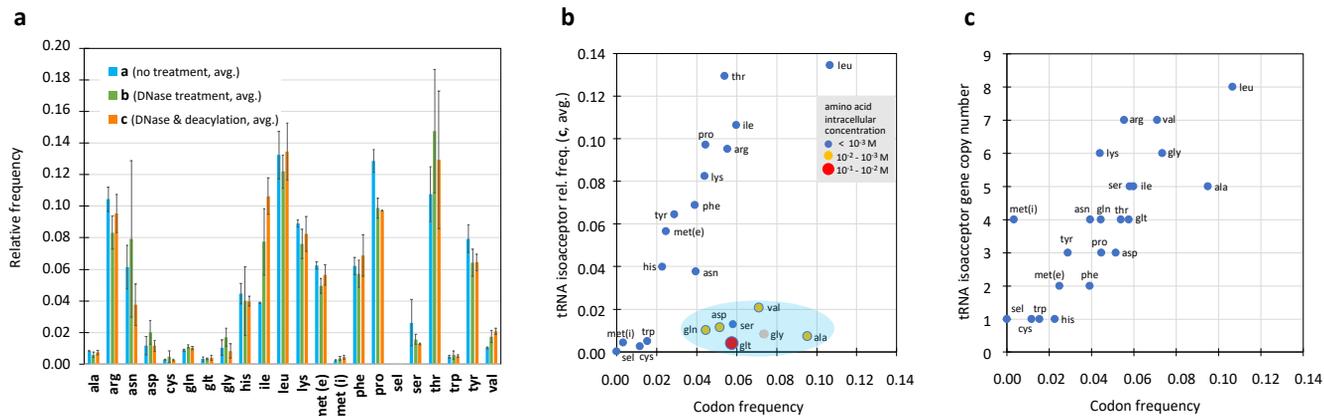


Figure 9. Relative quantification of *E. coli* CCA-ending tRNA isoacceptors (cultures grown in LB medium, sampled at OD_{650nm} about 0.4) and codon usage. a) Relative quantification of tRNA isoacceptors as established from the coverages at the 3' position of each tRNA (see Fig. 3a; values are in Supplemental File S6). Each bar represents the average relative frequency calculated over three replicates: a = <R1a, R2a, R3a>; b = <R1b, R2b, R3b>; c = <R1c, R2c, R3c>. b) Relative frequency of CCA-ending tRNA isoacceptors as a function of codon frequency in the *E. coli* K12 genome*. Inset: intracellular concentrations of encoded amino acids in *E. coli* as determined by Bennett et al. (2009). Cells grown under normal conditions, with glucose as the major source of carbon; cultures sampled at OD_{650nm} = 0.35. Glycine is the only encoded amino acid for which concentration was not determined in this study. c) *E. coli* MG1655 tRNA gene copy number as a function of codon frequency*.

*data from https://openwetware.org/wiki/Escherichia_coli/Codon_usage

316 amplified. Results of conventional deep sequencing indeed show that a significant proportion of tRNA 3' segments
317 lack the terminal adenosine (unpublished results), suggesting that the removal of this residue, that can be re-added
318 by the CCA-adding enzyme, may play a role in translation regulation. A tRNA quality control mechanism involving
319 these two enzymes has also been suggested (Wellner *et al.* 2018). The reason for which the relative frequency of
320 these seven CCA-ending isoacceptors is unexpectedly low might be related to the cellular concentration of their
321 cognate amino acid (Fig. 9b): in *E. coli* cells grown in conditions similar to that of our cultures, five of these amino
322 acids were found to be at least one order of magnitude more abundant than the other ones (Bennett *et al.* 2009),
323 while the amino acid of the most underrepresented isoacceptor, *glt*, is even 10^3 times more abundant in normal
324 growth conditions. High substrate concentration enables a fast turnover of tRNA aminoacylation, which might
325 abrogate the necessity for a large amount of functional isoacceptors. Further experiments are required to clarify this
326 issue.

327

328 **DISCUSSION**

329

330 Compared to recent tRNA sequencing proposals (Cozen *et al.* 2015; Zeng *et al.* 2015, Hauenschild *et al.* 2015;
331 Schwartz and Motorin 2017; Gogakos *et al.* 2017), the present method is relatively easier to implement because no
332 total RNA demethylase treatment and/or RNA fragmentation are required. Furthermore, we found that a
333 conventional RT enzyme (Superscript III) is processive enough to generate signals far to the 5' end of the tRNAs.
334 Avoiding fragmentation enables an accurate gene mapping of all reads, as they all start at the 3' end of the tRNA,
335 where essential identity elements are present. Two methodological aspects ensure the lowest possible bias: first, the
336 ligation of the 3' adapter is non-selective due to the invariant CCA^{3'} dangling end of mature tRNAs, that are specifically
337 amplified by GGT-ending primer during a first round of PCR. This feature of our protocol also allows to virtually
338 remove all background noise present in current protocols (Hauenschild *et al.* 2015, Clark *et al.* 2016, Gogakos *et al.*
339 2017). Second, TdT polyadenylation of cDNA after reverse transcription circumvents the issue of inefficient 5' tRNA
340 adapter ligation in conventional deep-sequencing protocols. Circularization of cDNA, which was used by Zheng *et al.*
341 (2015) in their own protocol, is another possibility. The obtained termination signals (**ts**) provide an atlas of the
342 modified state of all tRNAs (Fig. 4). Furthermore, we showed that two limitations inherent to deep-sequencing
343 experiments and the use of a conventional RT enzyme are simultaneously resolved by combining several samples: a
344 significant gain in the accuracy of **ts** signals is achieved, and these signals can be established down to the 3' end of
345 all tRNAs. The combined tRNA read coverage of the 9 samples reported in the present study, totalizing about 62
346 million mapped R2 reads, were just short of generating a full tRNA **ts** atlas: about 3% of sequence length was left
347 uncharacterized (magenta lines in Fig. 4). However, current standards of *Illumina* sequencing experiments generate
348 200 million reads. Based on the results of our computational processing, in which about 60% of the initial reads were
349 mapped, full tRNA characterization is almost certainly achievable with a single *Illumina* sequencing experiment. While
350 some modifications do not interfere with the RT enzyme, RNA chemical treatment may alter these silent

351 modifications and make them visible (Motorin et al. 2007), a procedure that has recently been applied by Zhang et
352 al. (2019) with an alternate protocol. Several silent modifications can still be detected when they occur nearby each
353 other (highlighted in orange in Fig. 4), implying that a change of the **ts** pattern can be observed if one modification is
354 missing. So far, near complete sets of tRNA modifications are available only for a couple of organisms in *Modomics*
355 (Boccaletto et al. 2018). Besides *Escherichia coli* (43 out of the 48 tRNAs of the *MG1655* strain are annotated), species
356 with exhaustive repertoire of tRNA modifications are *Streptomyces griseus*, *Haloferax volcanii*, *Mycoplasma*
357 *capricolum*, *Lactococcus lactis* and *Saccharomyces cerevisiae*. For most other species, alternate databases or specific
358 investigations may provide the missing information (supplemental Table S2). When no data is available, an extensive
359 amount of work will still be required to establish all tRNA modifications of the organism or organelle under
360 investigation. However, the potentially unlimited accuracy of the **ts** signal analysis outlined in the present work,
361 which only depends on the number of deep-sequenced samples, may allow the identification of tRNA positions with
362 altered modification patterns by comparing **ts** atlas of reference subjects to that of subjects with different genotype,
363 or exposed to different environmental conditions, or with different health or physiological condition(s). This would
364 allow to quickly narrow down the investigation to one or some modification enzymes that may be associated with
365 perturbed modification pattern(s) identified on some tRNAs (suppl. Fig. S6). Thus, while the present method may not
366 directly allow the identification of highlighted modifications, its power is to provide a robust and extensive diagnostic
367 of the modified state and relative frequencies of all cellular tRNAs with minimal benchwork

368

369

370 **ACKNOWLEDGEMENTS**

371 We thank Jinwei Zhang, Charles Bou-Nader, Sine Lo Svenningsen, Michael A. Sorensen and Shixin Ye for helpful
372 comments and suggestions on several aspects of our analysis, and Philippe Bouloc for providing lab space and a
373 stimulating working environment. Ji Wang was supported by the Chinese Scholarship Council, grant No.
374 201206140111.

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376

377 **AUTHORS CONTRIBUTION**

378 JL and DG conceived of the project. JW and FL performed the experimental work. JL, DG, JW, and CT interpreted the
379 results and performed the analysis. JL wrote the manuscript, with inputs from DG, CT and JW. CT and DG
380 implemented the computational pipeline. All authors read and approved the manuscript.

References

- Abe T, Inokuchi H, Yamada Y, Muto A, Iwasaki Y, Ikemura T. 2014. tRNADB-CE: tRNA gene database well-timed in the era of big sequence data. *Front Genet.* 5: 114.
- Alexandrov A., Chernyakov I., Gu W., Hiley S. L., Hughes T. R., Grayhack E. J., Phizicky E. M. 2006. Rapid tRNA Decay Can Result from Lack of Nonessential Modifications. *Mol. Cell* 21: 87–96.
- Baldrige KC, Jora M, Maranhao AC, Quick MM, Addepalli B, Brodbelt JS, Ellington AD, Limbach PA, Contreras, LM. 2018. Directed Evolution of Heterologous tRNAs Leads to Reduced Dependence on Post-transcriptional Modifications. *ACS Synthetic Biology* 7: 1315–1327.
- Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. 2009. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat Chem Biol.* 5: 593-599.
- Boccaletto P, Machnicka MA, Purta E, Piatkowski P, Baginski B, Wirecki TK, de Crécy-Lagard V, Ross R, Limbach PA, Kotter A, Helm M, Bujnicki JM. 2018. MODOMICS: a database of RNA modification pathways. 2017 update. *Nucleic Acids Res.* 4, 46(D1): D303-D307.
- Bohnsack MT, Sloan KE. 2018. The mitochondrial epitranscriptome: the roles of RNA modifications in mitochondrial translation and human disease. *Cell Mol Life Sci.* 75: 241-260.
- Chan C, Pham P, Dedon PC, Begley TJ. 2018. Lifestyle Modifications: Coordinating the tRNA Epitranscriptome With Codon Bias to Adapt Translation During Stress Responses. 2018. *Genome Biol.* 19: 228.
- Cozen AE, Quartley E, Holmes AD, Hrabeta-Robinson E, Phizicky EM, Lowe TM. 2015. ARM-seq: AlkB-facilitated RNA methylation sequencing reveals a complex landscape of modified tRNA fragments. *Nat Methods.* 12: 879-884.
- Czech A. 2020. Deep sequencing of tRNA's 3'-termini sheds light on CCA-tail integrity and maturation. *RNA* 26: 199-208.
- Dal Magro C, Keller P, Kotter A, Werner S, Duarte V, Marchand V, Ignarski M, Freiwald A, Müller RU, Dieterich C, Motorin Y, Butter F, Atta M, Helm M. 2018. A Vastly Increased Chemical Variety of RNA Modifications Containing a Thioacetal Structure. *Angew Chem Int Ed Engl.* 57: 7893-7897.
- de Crécy-Lagard V, Boccaletto P, Mangleburg CG, Sharma P, Lowe TM, Leidel SA, Bujnicki JM. 2019. Matching tRNA modifications in humans to their known and predicted enzymes. *Nucleic Acids Res.* 47: 2143-2159.
- Deutscher MP, Marlor CW, Zaniwski R. 1984. Ribonuclease T: new exoribonuclease possibly involved in end-turnover of tRNA. *Proc Natl Acad Sci* 81: 4290-4293.
- Deutscher MP, Marlor CW, Zaniwski R. 1985. RNase T is responsible for the end-turnover of tRNA in *Escherichia coli*. *Proc Natl Acad Sci* 82: 6427–6430.
- Dong H, Nilsson L, Kurland CG. 1996. Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rates. *J Mol Biol.* 260: 649-663.
- Du MZ, Wei W, Qin L, Liu S, Zhang AY, Zhang Y, Zhou H, Guo FB. 2017. Co-adaption of tRNA gene copy number and amino acid usage influences translation rates in three life domains. *DNA Res.* 24:623-633.
- Gogakos T, Brown M, Garzia A, Meyer C, Hafner M, Tuschl T. 2017. Characterizing Expression and Processing of Precursor and Mature Human tRNAs by Hydro-tRNAseq and PAR-CLIP. *Cell Rep.* 20: 1463-1475.
- Hauenschild R, Tserovski L, Schmid K, Thüring K, Winz ML, Sharma S, Entian KD, Wacheul L, Lafontaine DL, Anderson J, Alfonzo J, Hildebrandt A, Jäschke A, Motorin Y, Helm M. 2015. The reverse transcription signature of N-1 methyladenosine in RNA-Seq is sequence dependent. *Nucleic Acids Res.* 43: 9950-9964.
- Helm M, Motorin Y. 2017. Detecting RNA modifications in the epitranscriptome: predict and validate. *Nat Rev Genet.* 18: 275-291.
- Hengjiang Dong, Lars Nilsson and Charles G. Kurland. 1996. Co-variation of tRNA Abundance and Codon Usage in *Escherichia coli* at Different Growth Rates. *J. Mol. Biol.* 260: 649–663

- Higgs PG, Ran W. 2008. Coevolution of Codon Usage and tRNA Genes Leads to Alternative Stable States of Biased Codon Usage. *Mol Biol Evol.* 25: 2279-2291.
- Jonkhout N, Tran J, Smith MA, Schonrock N, Mattick JS, Novoa EM. 2017. the RNA modification landscape in human disease. *RNA* 23: 1754-1769.
- Krutyholowa R, Zakrzewski K, Glatt S. 2019. Charging the code - tRNA modification complexes. *Curr Opin Struct Biol.* 55: 138-146.
- Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 9: 357-359.
- Li Z, Deutscher MP. 2002. RNase E plays an essential role in the maturation of Escherichia coli tRNA precursors. *RNA* 8: 97-109
- Matthaei, J.H., Voigt, H.P., Heller, G., Neth, R., Schöch, G., Kübler, H., Amelunxen, F., Sander, G., and Parmeggiani, A. 1966. Specific interactions of ribosomes in decoding. *Cold Spring Harb. Symp. Quat. Biol.* 31: 25-38.
- Meyer B, Immer C, Kaiser S, Sharma S, Yang J, Watzinger P, Weiß L, Kotter A, Helm M, Seitz HM, Kötter P, Kellner S, Entian KD, Wöhnert J. 2020. Identification of the 3-amino-3-carboxypropyl (acp) transferase enzyme responsible for acp3U formation at position 47 in Escherichia coli tRNAs. *Nucleic Acids Research in press.*
- Mohanty BK, Maples VF, Kushner SR. 2012. Polyadenylation helps regulate functional tRNA levels in Escherichia coli. *Nucleic Acids Res* 40: 4589-4603.
- Motorin Y, Helm M. 2019. Methods for RNA Modification Mapping Using Deep Sequencing: Established and New Emerging Technologies. *Genes (Basel)* 10: 35.
- Motorin Y, Muller S, Behm-Ansmant I, Branlant C. 2007. Identification of modified residues in RNAs by reverse transcription-based methods. *Methods Enzymol.* 425: 21-53.
- Ng CS, Sinha A, Aniwah Y, Nah Q, Babu IR, Gu C, Chionh YH, Dedon PC, Preiser PR. 2018. tRNA epitranscriptomics and biased codon are linked to proteome expression in Plasmodium falciparum. *Mol Syst Biol.* 14: e8009.
- Novoa EM, Pavon-Eternod M, Pan T, Ribas de Pouplana L. 2012. A Role for tRNA Modifications in Genome Structure and Codon Usage. *Cell* 149: 202-213.
- Pollo-Oliveira L, de Crécy-Lagard V. 2018. Can Protein Expression Be Regulated by Modulation of tRNA Modification Profiles? *Biochemistry* 58: 355-362.
- Richter U, Evans ME, Clark WC, Marttinen P, Shoubridge EA, Suomalainen A, Wredenberg A, Wedell A, Pan T, Battersby BJ. 2018. RNA modification landscape of the human mitochondrial tRNA^{Lys} regulates protein synthesis. *Nature Communications* 9: 3966.
- Rykin P, Leung YY, Silverman IM, Childress M, Valladares O, Dragomir I, Gregory BD, Wang LS. 2013. HAMR: high-throughput annotation of modified ribonucleotides. *RNA* 19: 1684-1692.
- Shigematsu M, Honda S, Loher P, Telonis AG, Rigoutsos I, Kirino Y. 2017. YAMAT-seq: an efficient method for high-throughput sequencing of mature transfer RNAs. *Nucleic Acids Res.* 45: e70
- Schwartz S, Motorin Y. 2017. Next-generation sequencing technologies for detection of modified nucleotides in RNAs. *RNA Biology* 14: 1124-1137.
- Thorvaldsdóttir H, Robinson JT, Mesirov JP. 2013. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform.* 14: 178-192.
- Vandivier LE, Anderson ZD, Gregory BD. 2019. HAMR: High-Throughput Annotation of Modified Ribonucleotides. *Methods Mol Biol.* 1870: 51-67.
- Wellner K, Czech A, Ignatova Z, Betat H, Mörl M. 2018. Examining tRNA 3'-ends in Escherichia coli: teamwork between CCA-adding enzyme, RNase T, and RNase R. *RNA.* 24: 361-370.
- Zhang W, Eckwahl MJ, Zhou KI, Pan T. 2019. Sensitive and quantitative probing of pseudouridine modification in mRNA and long noncoding RNA 25: 1218-1225.

Zheng G, Qin Y, Clark WC, Dai Q, Yi C, He C, Lambowitz AM, Pan T. 2015. Efficient and quantitative high-throughput tRNA sequencing. *Nat Methods* 12: 835-837.

Figure Legends

Figure 1. Workflow of library preparation. See text for explanations and Supplemental File S1 for detailed protocol. Note that the stretch of 5 random positions (N5) preceding the poly(T) tail of the 5' adapter primer is a requirement of *Illumina* deep-sequencing spot localization.

Figure 2. Computational workflow of deep-sequencing *Illumina* data processing (See Supplemental Figure S1 for details). Shown values are from the R1a sample (all samples provided similar results). Among half a dozen tested strategies, the established workflow provided the sharpest termination signals. From the initial *QC-passed reads* (1), *trimmed reads* (2) were obtained with *cutadapt* by identifying and trimming all TdT-added poly-A (i.e. poly-Ts on the tRNA gene sequence) with at least 15 nt. In a second step, a local mapping of the reads onto the *E. coli* genome with *bowtie2* (Langmead and Salzberg 2012) was achieved, providing the *mapped reads* (3). The *-local* option maximizes the stretch of matching bases by removing mismatches at the end of the reads (softclipping). In a third step, mapped reads were filtered with a custom *awk* script to select only the reads ending with *CCA/TGG* (depending to the orientation of the tRNA on the genomic sequence), thus complementing the selection of mature tRNA achieved during library preparation. This operation provided the *final mapping* (4), from which a *wig* file (5) compiling the coverage of all expressed RNA genes was established with *samtools depth* using the *-d 1000000* and *-a* options.

Figure 3. Overview of the analysis of deep-sequenced CCA^{3'}-amplified RNA transcripts. **a)** Snapshot of an *IGV* window (Thorvaldsdóttir et al. 2013) centered on the *lysQ* tRNA gene of *E. coli* MG1655 genome, showing the coverage established from the R1b sample. This gene is oriented 5'-3' from left to right. Interruption of reverse transcription events (from 3' to 5') generate coverage jumps. **b)** Termination signals (**ts**, defined in the box) of *lysQ* tRNA, established from the coverage of R1b, R2b and R3b samples. Because the *lysQ* sequence is identical to that of *lysT*, *lysV*, *lysW*, *lysY* and *lysZ*, reads are evenly distributed among these 6 genes. Base modifications symbols indicated below the sequence are from the *Modomics* database (Boccaletto et al. 2018) (Supplemental Table S1). **c)** **Ts-ts** Plots illustrating the lowest (left) and the highest (right) obtained Pearson correlation coefficients (*r*) between samples (see Table 3); *n* = 3146 in both plots. **d)** Pearson correlation coefficients as a function of the difference in total tRNA read number between samples.

Figure 4. Gain in precision obtained in the **ts** analysis when **ts** signals from 3 samples are averaged, as compared to signals obtained from single samples (illustration with *valW* tRNA). **a)** Comparison of **ts** values between three biological replicates (R1, R2 and R3) with a single sample (**c** treatment). **b)** Same comparison as in **a**, but with **ts** values of each biological replicate averaged over three samples (**a**, **b** and **c**). Because RNA sample treatment (**a**, **b**, **c**) were found not to noticeably alter **ts** signals (see Table 3 and text), they are considered as equivalent. For a global quantitative analysis, see Table 4.

Figure 5. Atlas of **ts** signals of all *E. coli* tRNAs, with base modifications reported from *Modomics* (as of Nov. 2017), with one correction: only one **D** modification could be attributed to *serW* (*serX*). *Modomics*-uncharacterized tRNAs are underlined with gray lines. Identical tRNA genes are represented only once (copies are listed in brackets). **Ts** signals are established from the combined coverage of all 9 samples of the present work (Table 2). Magenta lines highlight 5' portions of tRNAs for which the total coverage was too low to establish **ts** signals. Plots with individual **ts** values for all 9 samples are available in Supplemental File S4.

Figure 6. **a)** Standard tRNA nucleotide numbering according to the *tRNA gene database curated by experts* website (Abe *et al.*, 2014). The colored regions correspond to the four successively analyzed tRNA segments, from 3' to 5': green, red, sand and blue. **b)** **Ts** analysis from pos. 76 to pos. 48 (green segment), established from an alignment of all tRNA transcripts. The sample with the highest values (R3c) is highlighted with a thick line. The left inset illustrates individual tRNA values (and the average) of the R3c sample. The right inset shows the sum of the **ts** signal as a function of the number of (G+C) residues in positions 61 to 65. Each blue dot corresponds to a tRNA; values averaged over all 9 samples (see Table 1). Red dots correspond to values averaged over all tRNAs. The base conservation logo reported at the bottom is based on all *E. coli* tRNA listed in *Modomics*. Because T and P modifications correspond to a stretch of two As on the cDNA, the **ts** signal of the two residues essentially combine into a single signal on the P residue (blue arrow) (see Material and Methods). **c)** **Ts** analysis of the variable loop (red segment). **Ts** signal in pos. 47 for all tRNAs bearing the **7** modification in pos. 46, according to *Modomics*. For tRNAs with multiple gene copies (e.g. *asnT*, *asnU*, *asnV*, *asnW*), only one set of values is indicated. tRNA genes are ranked from left to right following an increasing average value of the **ts** signal. The vertical dashed line splits tRNAs into those with U (left) from those with **X** in pos. 47 according to *Modomics* (as of Nov. 2017). Insets illustrate three **ts** signals. **d)** Sum of **ts** values from pos. 40 to 46 (trailing **ts** signals) as a function of the **ts** value in pos. 47. Note that according to the *Modomics* database, all *E. coli* tRNAs with **7** and **X** modifications do not have any *e* residue in the variable loop, implying that the sequences analyzed in **c** and **d** follow standard numbering (panel **a**).

Figure 7. Ts analysis of the anticodon loop. Plots of the average values and standard deviations established from all 9 samples (Table 1), highlighting ts signatures of some typical *E. coli* combinations of modifications (panels a to i) and their ts error that occur when only a single sample is considered. Anticodons are boxed in magenta. Known modifications are reported from the *Modomics* database below the sequences (5'-3'). Highlighted modifications (in orange and red) are illustrated above in the same order (cartoons are from the *Modomics* website). A blue arrow indicates a shift of a ts signal associated with a modification as a result of the presence of U residue(s) on its 3' side (highlighted in blue), an artifact caused by poly-T computational trimming (see Material and Methods). Red arrows highlight the shift of the ts signal to the 5' direction observed with the 6 modification. The ts signal could not be established on the 5' side of the } modification for *IleX* (panel d, dashed lines) in all samples. Although Inosine (I) is associated with a low ts signal (panel i), an examination of the read coverage shows that it leads to incorporation of 86%C and 14%U on the cDNA, a typical signal of the presence of this modification (Schwartz and Motorin, 2017).

Figure 8. Ts analysis of the D-loop. **a)** Illustration of the effect of the presence of ≥ 3 (*hisR*, on top) and < 3 (*asnT*, bottom) dihydrouridine modifications. The curly brackets encompassing a window of 11 nts are centered on every reported dihydrouridine modification (this size allows to include most of the associated ts signal). **b)** Plot of the sum of ts signals in the window with the highest signal (*) as a function of the number of reported dihydrouridine modifications. **c)** Plots of the number of uridine(s) modified into dihydrouridine(s) in the D-loop according to the *Modomics* database (nov. 2017). In two cases, with 3 and 4 uridines (*argU* and *glyT*), no such modification has been reported. **d)** Ts signal and sequence similarity (in green) between *leuU*, *leuP*, *leuQ*, *leuT*, *leuV*, for which three dihydrouridines have been reported, and *leuW*, for which the ts analysis (in b) and sequence similarity predict the presence of a third unlisted dihydrouridine (circled). The low ts signal of *glyT* is consistent with the absence of at least two dihydrouridine modifications among the four uridines of the loop. Ts values are established from the combined coverage of all 9 samples (Table 1). D-loop segments are highlighted with a blue line.

Figure 9. Relative quantification of *E. coli* CCA-ending tRNA isoacceptors (cultures grown in LB medium, sampled at $OD_{650nm} \approx 0.4$) and codon usage. **a)** Relative quantification of tRNA isoacceptors as established from the coverages at the 3' position of each tRNA (see Fig. 3a; values are in Supplemental File S6). Each bar represents the average relative frequency calculated over three replicates: **a** = <R1a, R2a, R3a>; **b** = <R1b, R2b, R3b>; **c** = <R1c, R2c, R3c>. **b)** Relative frequency of CCA-ending tRNA isoacceptors as a function of codon frequency in the *E. coli* K12 genome*. Inset: intracellular concentrations of encoded amino acids in *E. coli* as determined by Bennett *et al.* (2009). Cells grown under normal conditions, with glucose as the major source of carbon; cultures sampled at $OD_{650nm} = 0.35$. Glycine is the only encoded amino acid for which concentration was not determined in this study. **c)** *E. coli* MG1655 tRNA gene copy number as a function of codon frequency*.

*data from https://openwetware.org/wiki/Escherichia_coli/Codon_usage