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► **To cite this version:**

Virginie Marchand, Lilia Ayadi, Aseel El Hajj, Florence Blanloeil-Oillo, Mark Helm, et al.. High-Throughput Mapping of 2'-O-Me Residues in RNA Using Next-Generation Sequencing (Illumina RiboMethSeq Protocol). *Methods in Molecular Biology*, 2017, 1562, pp.171-187. 10.1007/978-1-4939-6807-7_12. hal-01799289

HAL Id: hal-01799289

<https://hal.univ-lorraine.fr/hal-01799289v1>

Submitted on 11 Mar 2022

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High-throughput mapping of 2'-O-Me residues in RNA using next-generation sequencing (Illumina RiboMethSeq protocol)

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Mapping and quantification of 2'O-Me by RiboMethSeq

Summary

Detection of RNA modifications in native RNAs is a tedious and laborious task, since the global level of these residues is low and most of the suitable physico-chemical methods require purification of the RNA of interest almost to homogeneity. To overcome these limitations, methods based on RT-driven primer extension have been developed and successfully used, sometimes in combination with a specific chemical treatment. Nowadays, some of these approaches have been coupled to high-throughput sequencing technologies, allowing the access to transcriptome-wide data. RNA 2'-O-methylation is one of the ubiquitous nucleotide modifications found in many RNA types from bacteria, archaea and eukarya. Here, we describe a reliable and optimized protocol based on alkaline fragmentation of total RNA coupled to a commonly used ligation approach followed by Illumina sequencing. We describe the methodology for detection and relative quantification of 2'-O-methylations with a high sensitivity and reproducibility even with a limited amount of starting material (1 ng of total RNA). Altogether this technique unlocks a technological barrier since it will be applicable for routine parallel treatment of biological and clinical samples to decipher the functions of 2'-O-methylations in pathologies.

Key words: 2'-O-Methylation; high-throughput sequencing; RNA modification; ribose methylation; alkaline fragmentation

1. Introduction

One very common and widespread modification in RNA is a methylation of the ribose 2'-OH

moiety, which was found in tRNA, rRNA, snRNA, mRNA as well as in piRNA and miRNA (1–5).

Three major approaches have been proposed for detection of 2'-O-methylated residues in mature cellular RNAs. Some of them have been extensively used in the past (e.g. reverse transcription (RT) at low [dNTP] or RT on randomly fragmented RNAs) and allowed the establishment of 2'-O-methylation profiles for rRNAs from various species and also for some snRNAs (6,7). Detection and even quantification of 2'-O-methylation in RNA was achieved using specific RNase H cleavage of 2'-O-methyl RNA-DNA hybrids and by dedicated ligation-based techniques (8,9). More recently, the combination of RT at low concentration of dNTPs ([dNTP]) with quantitative PCR was used for relative quantification of 2'-O-Me residues (10). Similarly, a direct qRT-PCR detection method using a special mutant of thermostable DNA polymerase with high RT-activity was described recently (11). However, all these methods are extremely laborious and time-consuming and only allow site-by-site detection and relative measurement of 2'-O-methylations. In some instances, bioinformatics approaches for 2'-O-Me mapping can also be employed, which resulted, for example, in the deduction of ribose methylation patterns of rRNAs in various archaea after careful identification of C/D-box sRNA guides (12).

A high-throughput approach for 2'-O-Me detection based on random RNA fragmentation under alkaline conditions and library preparation followed by next-generation sequencing (NGS) was proposed last year (13). The phosphodiester bond at the 3' of 2'-O-Me nucleotides is relatively resistant to nucleolytic cleavage, and thus the RNA fragments starting or ending at the nucleotide at the +1 position to the modified residue are excluded from a randomly fragmented library. However, the suggested approach was based on a rather non-conventional ligation procedure with homemade 3'-cyclophosphate modified adapters and particular RNA ligase mutant (13).

Here we provide a detailed description and optimization of the RiboMethSeq protocol based on simple alkaline hydrolysis coupled to a standard library preparation protocol suitable for Illumina sequencing (**Fig. 1**). The RiboMethSeq protocol can be robustly applied to detection and even relative quantification of 2'-O-Me sites in highly abundant or enriched RNAs and has an acceptable performance with a very small amount of starting RNA material (~1 ng).

2. Materials

Prepare all solutions using RNase-free water. Wear gloves to prevent degradation of RNA samples by RNases.

2.1. RNA quantification and quality assessment

2.1.1. RNA quantification

1. UV-visible small volume spectrophotometer: any kind of UV visible spectrophotometer allowing measurements of 1 μ l samples.
2. 1.5 ml RNase-free microcentrifuge tubes.
3. RNase-free water.

2.1.2. RNA quality assessment

4. Agilent 2100 Bioanalyzer or 2200 TapeStation (Agilent Technologies) or Experion (BioRad) or LabChip GX (Caliper). We use an Agilent 2100 Bioanalyzer.
5. Agilent RNA 6000 Pico kit (quantitative range 50-5,000 pg/ μ l) (see **Note 1**).
6. Chip priming station (Agilent Technologies).
7. RNase-free 1.5 ml microcentrifuge tubes.
8. RNase-free water.

2.2. Alkaline Hydrolysis and RNA fragmentation quality control

2.2.1. Alkaline Hydrolysis

1. Sodium bicarbonate buffer: 100 mM, pH9.2.
2. RNase-free water.
3. Individual RNase-free 0.2 ml PCR tubes.
4. PCR Thermal cycler (we use Agilent SureCycler 8000).
5. RNase-free 1.5 ml microcentrifuge tubes.
6. Absolute ethanol.
7. GlycoBlue Coprecipitant: 15 mg/ml (Ambion).
8. NaOAc: 3M in water, pH5.2.
9. Dewar containing liquid nitrogen.

2.2.2. RNA fragmentation quality control

10. Agilent 2100 Bioanalyzer (Agilent Technologies).
9. Agilent RNA 6000 Pico kit (quantitative range 50-5,000 pg/ μ l) (see **Note 1**).
11. Chip priming station (Agilent Technologies).
12. RNase-free 1.5 ml microcentrifuge tubes.

2.3. End-repair

1. RNase-free water.
2. Antarctic Phosphatase: 5U/ μ l (New England Biolabs).
3. T4 PNK: 10U/ μ l (New England Biolabs).
4. ATP: 10 mM.
5. RiboLock RNase Inhibitor: 40U/ μ l.
6. 0.2 ml RNase-free PCR tubes, strips of 8.
7. Flat PCR Caps, strips of 8.
8. PCR thermal cycler (we use Agilent SureCycler 8000).

2.4. RNA purification

1. RNeasy MinElute Cleanup kit.
2. Absolute Ethanol.
3. 80% Ethanol.

2.5. Library preparation using NEBNext® Multiplex Small RNA Library Prep Set for Illumina®

1. NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (set 1 or 2, New England Biolabs) (See **Note 2**).
2. 0.2 ml PCR tubes, strips of 8.
3. Flat PCR Caps, strips of 8.

2.6. Library purification using GeneJET PCR Purification kit

1. GeneJET PCR Purification kit or equivalent.
2. 1.5 ml DNA low-binding tubes.

2.7. Library quantification and quality assessment

2.7.1. Library quantification

1. Any kind of fluorometer able to quantify DNA library with high sensitivity (e.g., Qubit® 2.0 fluorometer).
2. Qubit® dsDNA HS Assay kit (0.2-100 ng).
3. Thin-walled polypropylene tubes of 500 µl compatible with the fluorometer (e.g., Qubit® Assay Tube or Axygen® PCR-05-C tubes).

2.7.2. Library quality assessment

1. Agilent 2100 Bioanalyzer (Agilent Technologies).

2. Agilent HS DNA kit (quantitative range 5-500 pg/ μ l).
3. Chip priming station (Agilent Technologies).
4. RNase-free 1.5 ml microcentrifuge tubes.

2.8. Library sequencing

1. Any kind of Illumina sequencers (starting from Miseq to Hiseq).
2. Any appropriate sequencing kit for a single read length of 35-50 nt.

2.9. Bioinformatic analysis

1. Unix (Linux) server (we are using Illumina Compute Dell server).
2. Adapter trimming software Trimmomatic (current version 0.36
<http://www.usadellab.org/cms/?page=trimmomatic>).
3. Alignment software Bowtie 2.0 (current version 2.2.9 <http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>).
4. Microsoft Excel or Open(Libre)Office calc spreadsheet software.

3. Methods

3.1. RNA quantification and quality assessment

Carry out all procedures at room temperature.

3.1.1. RNA quantification

1. On a Nanodrop 2000 start screen; select the “Nucleic Acid” application.
2. After the wavelength verification test, select the type of sample to measure, in this case “RNA”.
3. Prepare the blank: the buffer/solution used for sample resuspension but without any trace of RNA (e.g., RNase-free water).

4. Load 1 μl of the blank solution to the bottom pedestal, lower the arm and click on the “Blank” button.
5. Wipe the upper and lower pedestal using a dry wipe and load 1 μl of one of your samples of interest to the bottom pedestal, lower the arm, and click “Measure”.
6. Analyze the data obtained for your different RNA samples. For “pure” RNAs, the ratio A260/A280 should be 2, the ratio A260/A230 should be in the range of 1.8-2.2 (see **Note 3 and Note 4**).

3.1.2. RNA quality assessment

1. Before starting the experiments, equilibrate all solutions of the kit at room temperature for at least 30 min in the dark. Vortex them and spin them down before use.
2. Transfer 550 μl of gel matrix (red cap vial) into a spin filter provided in the kit.
3. Centrifuge for 10 minutes at 1,500xg at room temperature.
4. Prepare 65 μl aliquots of the gel and store them at 4°C for a maximum of one month.
5. Prepare the gel-dye mix by mixing 1 μl of RNA dye concentrate to a gel aliquot.
6. Centrifuge for 10 minutes at 13,000xg at room temperature.
7. Dilute your RNA samples quantified on Nanodrop to 3-5 ng/ μl with RNase-free water to be within the optimal range concentration of the assay.
8. Add 1 μl of your diluted RNA samples to 11 different 1.5 ml tubes already containing 5 μl of RNA marker (green cap vial) (see **Note 5**). Mix by pipetting up and down.
9. Mix 1 μl of the ladder (see **Note 6**) with 5 μl of RNA marker (green cap vial). Mix by pipetting up and down.
10. Prepare the chip priming station. Adjust the syringe clip to the highest top position.
11. Load 9 μl of the gel-dye mix in the well marked with a “G” surrounded by a black circle.

12. Close the chip priming station properly and press the plunger of the syringe until it is held by the clip.
13. Wait for 30 sec and then release the clip.
14. Wait for 5 sec until the plunger stops and pull it slowly back to the 1 ml position of the syringe.
15. Open the chip priming station and load 9 μ l of the gel-dye mix in the 2 other wells marked "G".
16. Load 9 μ l of the conditioning solution (white cap vial) in the well marked "CS".
17. Load 6 μ l of the diluted ladder in the well marked with a ladder.
18. Load 6 μ l of the diluted RNA samples in the wells marked 1-11.
19. Inspect the chip and make sure that no liquid spill is present on the edges of the wells.
20. Insert the chip in the Agilent 2100 Bioanalyzer and close the lid (see **Note 7**).
21. Select the following assay "Eukaryote Total RNA Pico series II" in the 2100 expert software screen.
22. Press "Start" to begin the chip to run (see **Note 8**).
23. After the run, immediately remove the chip and clean the electrodes with the electrode cleaner filled with 350 μ l of RNase-free water.
24. Analyze the results of the chip (**Fig. 2**).

3.2. Alkaline Hydrolysis and RNA fragmentation quality control

3.2.1. Alkaline Hydrolysis

1. Prepare one 1.5 ml tube per sample to be analyzed ("precipitation tube") containing 10 μ l of NaOAc, 1 μ l of glycoblue and 1 ml of absolute ethanol for subsequent precipitation of the sample (store at -20°C until further use).
2. Dilute your RNA samples to a concentration of 20 ng/ μ l with RNase-free water.

3. To individual PCR tubes, add 5 μ l of each of your diluted RNA samples (see **Note 9**), keep on ice until further use.
4. Add 5 μ l of bicarbonate buffer and mix by pipetting up and down.
5. Incubate in a thermal cycler pre-heated at 95°C. Start a timer and incubate for 4-12 minutes (see **Note 10**).
6. Proceed with the next sample every 30 sec.
7. Stop each reaction after the required time at 95°C by spinning down the PCR microtube and add the whole sample into the corresponding 1.5 ml precipitation tube from step 1.
8. Mix by inverting the tube several times and throw it into liquid nitrogen.
9. Recover it from the liquid nitrogen and centrifuge your samples for 30 min at 4°C at full speed in a microcentrifuge.
10. Take out the supernatant and make sure not to lose the pellet.
11. Wash with 600 μ l of 80% ethanol.
12. Centrifuge your samples for 10 min at 4°C at full speed.
13. Take out the supernatant.
14. Centrifuge your samples for a short spin.
15. Remove any liquid left.
16. Incubate your samples with open lid for 2 min at 37°C and 5 min at room temperature.
17. Resuspend the pellet with 20 μ l of RNase-free water.

3.2.2. RNA fragmentation quality control

1. Prepare your samples by mixing 5 μ l of RNA marker (green cap vial) with 1 μ l of your fragmented RNA samples.
2. With the rest of gel-dye mix, load 9 μ l in the well marked “G” surrounded with a black circle and proceed as described in section “RNA quality control” from step 12 to

18 from section 3.1.2.

3. Analyze the results obtained (**Fig. 2**).

3.3. End-repair

1. Combine 16 μl of your treated RNA samples in a PCR tubes with 2 μl of phosphatase buffer, 1 μl of RiboLock RNase Inhibitor and 1 μl of Antarctic Phosphatase.
2. Mix by pipetting up and down.
3. Incubate the PCR tubes for 30 min at 37°C and then for 5 min at 70°C (to inactivate the phosphatase) and store for indefinite hold at 4°C in a thermal cycler.
4. Add the following components to the previous mix: 17 μl of RNase-free water, 5 μl of PNK buffer, 5 μl of ATP (see **Note 11**), 1 μl of RiboLock RNase Inhibitor, 2 μl of PNK enzyme.
5. Incubate in a thermal cycler for 1h at 37°C and immediately proceed to the next step.

3.4. RNA purification

All the reagents except ethanol used for RNA purification are part of RNeasy MinElute Cleanup kit.

1. Transfer the sample to a new 1.5 ml tube, and to adjust the final volume to 100 μl , add 50 μl of RNase-free water.
2. Add 350 μl of RLT buffer, mix by vortexing.
3. Add 675 μl of absolute ethanol and mix by inverting the tube up and down (see **Note 12**).
4. Transfer 700 μl of the sample to an RNeasy MinElute spin column (stored at 4°C until use). Centrifuge for 30 sec at 8,000xg.
5. Repeat the step 4 with the rest of the sample. Then, add 500 μl of RPE buffer to the

column. Centrifuge for 30 sec at 8,000xg.

6. Discard the flowthrough. Add 750 μ l of 80% Ethanol. Centrifuge for 2 min at 8,000xg.
7. Transfer the column to a new collection tube and centrifuge at full speed for 5 min with the lid open.
8. Transfer the column to a new 1.5 ml tube (provided with the kit). Add 10 μ l of RNase-free water in the center of the column filter. Wait for 1 min.
9. Centrifuge at full speed for 1 min to elute. The recovered volume is about 9 μ l.

3.5. Library preparation using NEBNext® Multiplex Small RNA Library Prep Set for Illumina®

1. Mix 6 μ l of RNA sample with 1 μ l of 3'SR adaptor (green cap vial) in a PCR tube.
2. Incubate for 2 min at 70°C in a preheated thermal cycler. Transfer immediately to ice.
3. Add 10 μ l of 3' Ligation Buffer (green cap vial) and 3 μ l of 3' Ligation Enzyme (green cap vial).
4. Incubate for 1 hour at 25°C in a thermal cycler.
5. Add 4.5 μ l of RNase-free water and 1 μ l of SR RT primer (pink cap vial).
6. Incubate for 5 min at 75°C, 15 min at 37°C and 15 min at 25°C.
7. Within the last 15 min of incubation, add $1.1 * n$ (n =number of samples) μ l of the 5' SR adaptor (yellow cap vial) in an individual PCR tube (previously resuspended in 120 μ l of RNase-free water and stored at -80°C).
8. Denature the 5'SR adaptor in a thermal cycler for 2 min at 70°C and immediately place the tube on ice (see **Note 13**).
9. Add 1 μ l of 5'SR adaptor (previously denatured), 1 μ l of 5'Ligation Reaction Buffer (yellow cap vial) and 2.5 μ l of Ligase Enzyme Mix (yellow cap vial).

10. Incubate for 1h at 25°C in a thermal cycler.
11. Add the following components to the adaptor ligated RNA mix from the previous step: 8 µl of First strand synthesis reaction buffer (red cap vial), 1 µl of Murine RNase inhibitor (red cap vial), 1 µl of ProtoScript II reverse transcriptase (red cap vial) and mix well by pipetting up and down.
12. Incubate for 1h at 50°C.
13. Immediately proceed to PCR amplification (see **Note 14**). Add the following components to the RT reaction mix from the previous step: 50 µl of LongAmp Taq Master Mix (bleu cap vial), 2.5 µl of SR primer (blue cap vial), 2.5 µl of index primer (see **Note 15**), and 5 µl of RNase-free water. Mix well.
14. Perform the following PCR cycling conditions: 1 cycle of initial denaturation for 30 sec at 94°C, 12-15 cycles of denaturation 15 sec at 94°C, annealing 30 sec at 62°C, extension 15 sec at 70°C, 1 cycle of final extension for 5 min at 70°C and store at 4°C for indefinite hold.

3.6. Purification of the library using GeneJET PCR Purification kit

1. Transfer the PCR mix to a 1.5 ml tube, and add 100 µl of binding buffer. Mix thoroughly.
2. Transfer the solution to the purification column. Centrifuge at full speed for 30 sec. Discard the flowthrough.
3. Add 700 µl of wash buffer to the column and centrifuge at full speed for 30 sec. Discard the flowthrough.
4. Centrifuge the empty column for 1 additional min.
5. Transfer the column to a clean 1.5 ml DNA low binding tube. Add 30 µl of Elution buffer to the center of the column membrane and centrifuge at full speed for 1 min.

6. Store the purified library at -20°C until further use.

3.7. Library quantification and quality assessment

3.7.1. Library quantification

1. Before starting the experiments, incubate all solutions of the Qubit dsDNA HS assay kit at room temperature for at least 30 min. The kit provides the concentrated assay reagent, dilution buffer and pre-diluted standards.
2. Prepare the dye working solution by diluting the concentrated assay reagent 1:200 in dilution buffer. Prepare 200 µl of working solution for each sample and two additional standards.
3. Prepare the two standards annotated “C” and “D” by mixing 10 µl of standard with 190 µl of working solution.
4. Add working solution to 1 µl of RNA sample to obtain 200 µl in total.
5. Vortex the tubes for 2 seconds and incubate them for 2 min at room temperature.
6. Insert the tubes into the Qubit[®] 2.0 Fluorometer and proceed with measurements: on the home screen of the Qubit[®] 2.0 Fluorometer, choose the type of assay (e.g. “HS DNA”) for which you want to perform a new calibration.
7. Press “Yes” to read new standards.
8. When indicated, insert the standard tube and press “Read”. Standard #1 and #2 correspond to standards “C” and “D”, respectively.
9. Once the calibration is done, insert each sample and press “Read” to make the measurements. Check that the value of your samples is within the assay's range, and press “Calculate Stock Conc” (see **Note 16**).

3.7.2. Quality assessment

1. Before starting the experiments, incubate all solutions of the Agilent High Sensitivity DNA kit at room temperature for at least 30 min in the dark. Vortex them and spin them down before use.
2. Add 15 μ l of High sensitivity DNA dye concentrate (blue cap vial) into a High Sensitivity DNA gel matrix vial (red cap vial).
3. Vortex for 10 seconds and transfer the gel-dye mix to the center of the spin filter.
4. Centrifuge for 10 minutes at 2,240xg (see **Note 17**).
5. Add 1 μ l of each of your library to 11 different tubes of 1.5 ml already containing 5 μ l of RNA marker (green cap vial). Mix by pipetting up and down.
6. Mix 1 μ l of the ladder (yellow cap vial) with 5 μ l of High sensitivity DNA marker (green cap vial). Mix by pipetting up and down.
7. Prepare the chip priming station. Adjust the syringe clip to the lowest top position.
8. Load 9 μ l of the gel-dye mix in the well marked with a “G” surrounded by a black circle.
9. Close the chip priming station properly and press the plunger of the syringe until it is held by the clip.
10. Wait for 1 min and then release the clip.
11. Wait for 5 sec. until the plunger stops and pull it slowly back to the 1 ml position of the syringe.
12. Open the chip priming station and load 9 μ l of the gel-dye mix in the 3 other wells marked “G”.
13. Load 6 μ l of the diluted ladder in the well marked with a ladder.
14. Load 6 μ l of the diluted library samples in the wells labeled 1-11.
15. Insert the chip in the Agilent 2100 Bioanalyzer, close the lid and select the following assay “High Sensitivity DNA” in the 2100 expert software screen.

16. Press “Start” to begin the chip to run.
17. After the run, immediately remove the chip and clean the electrodes with the electrode cleaner filled with 350 µl of RNase-free water.
18. Analyze the results of the chip (**Fig. 3**).

3.8. Library sequencing

1. For sequencing, libraries are multiplexed and diluted to 8-12 pM final concentration.
2. Sequencing depth or coverage depends on the length of target RNA and can roughly be estimated at 1,000 reads per RNA nt.
3. Sequencing length may vary from 35 to 50 nt in a single read mode.

3.9. Bioinformatic analysis

1. Trim adapter sequences of raw reads (FastQ files) using Trimmomatic with the following parameters : `java -jar trimmomatic-0.35.jar SE -phred33 input.fq.gz output.fq.gz ILLUMINACLIP:TruSeq3-SE:2:30:10 LEADING:30 TRAILING:30 SLIDINGWINDOW:4:15 AVGQUAL:30 MINLEN:17` (see **Note 18**).
2. Align the trimmed reads to the appropriate reference sequence (yeast or human rRNA for example) using bowtie2 with the following parameters : `bowtie2 --sensitive -L 17 -x <bt2-idx> -U <r> -S.`
3. Count the 5'-ends in the produced *.sam file using awk command : `awk '{print $4}' *.sam | sort | uniq -c | awk '{print $3,$2,$1,$4}' | sort -n.`
4. Import the file containing 5'-end counts into Excel or Calc.
5. Calculate ScoreMax for each position, make a ratio of number of 5'-reads ends between preceding and following position and calculate ScoreMAX as a ratio of a drop for a given position compared to the average for 12 neighboring positions (-6/+6).
6. Calculate the RiboMethScore, use the following formula : $\text{RiboMethScore} = 1 -$

$n_i / (0.5 * (\text{SUM}(n_j * W_j) / \text{SUM}(W_j) + \text{SUM}(n_k * W_k) / \text{SUM}(W_k)))$, where n_i – 5'-end count for a given position, j – varies from $i-6$ to $i-1$, k varies from $i+1$ to $i+6$, Weight parameters are defined as following $W_j = 1 - 0.1 * (j-1)$ idem for W_k . Thus, W_j or W_k parameter varies from 0.5 for positions $-6/+6$ to 1 for positions $-1/+1$.

4. Notes:

1. If the size of your RNA to be fragmented is below 200 nts, it is appropriate to use Agilent Small RNA kit (quantitative range 50-2,000 pg/ μ l) following manufacturer's recommendations.
2. The kit NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (set 1) (NEB, E7300S) includes a set of 12 barcoding primers (numbered 1-12) that will be used for multiplexing reactions during PCR amplification. There is also a version set 2 with primers (numbers 13-24). If you do not need these barcoding primers, you may order a similar kit without the primers and use any other source of barcoding primers (Illumina, Epicentre, NEB).
3. If your RNA sample is diluted with RNase-free water instead of 10 mM Tris-EDTA (TE) pH8.0, the ratio A260/A280 may be below 2.0 due to the lower pH of water (14). A ratio A260/A280 of 1.8 for samples diluted in RNase-free water is considered "pure" for RNA.
4. If your RNA sample is contaminated by phenol or chaotropic salts (e.g. guanidinium thiocyanate used in Trizol® extraction or other protocols), this will result in a ratio A260/A230 below 1.8. Another round of Phenol-Choroform-Isoamyl Alcohol (PCA) extraction and two successive steps of chloroform extraction followed by ethanol precipitation are in this case recommended before alkaline digestion.
5. In case you are working with less than 11 samples, in the empty wells replace RNA with 1 μ l of RNase-free water.

6. The ladder loaded in the Pico RNA chip is provided in a separate package and may be prepared before the experiment: spin down the tube and transfer 10 μ l to a RNase-free tube. Heat for 2 min at 70°C. Cool down on ice and add 90 μ l of RNase-free water. Prepare 5 μ l aliquots using the Safe-Lock PCR tubes provided in the kit and store them at -70°C. Before use, thaw one tube and keep it on ice. The ladder is quite stable at -70°C and may be used at least 4 months.
7. RNase contamination problems of the Bioanalyzer electrodes are very frequent and will affect the RNA integrity number of your samples. Therefore, if the Agilent 2100 Bioanalyzer is also frequently used to run DNA chips, it is strongly recommended to use a dedicated electrode cartridge only for RNA assays. In addition, we recommend for each chip to load an internal RNA control (total RNA preparation with a known RIN>9). If you encounter contamination problems, soak the electrode cartridge into an RNaseZap® decontamination solution (Ambion) for at least 10 min, then rinse the electrodes with RNase-free water and let them dry out for at least one night.
8. The Agilent 2100 Bioanalyzer is very sensitive to vibrations and this may affect your results. Therefore make sure that no vibrations will occur during the run.
9. The RNA quantity may be decreased to a minimal starting amount of 5-10 ng without considerably affecting coverage and calculation of the RiboMethScore. However, if you are aiming to discover new 2'-O-Me sites in an unknown RNA, we recommend using 100-250 ng as starting material.
10. If total RNA samples (from yeast, human, bacteria) are of good quality (RIN>8), fragmentation time is around 8-10 min. However this time may be decreased with RNA of poor quality (4-6 min) or may be increased with RNA species of higher stability (ie: tRNAs) (12-14 min) (**Fig. 2**). We recommend testing 3 to 4 different times of fragmentation to define the appropriate conditions for hydrolysis. In general,

For long RNAs, the optimal size distribution is around 50-100 nt, while for short RNAs (>200 nts), 20-50 nt is the appropriate size distribution.

11. We tried different ATP concentrations ($1 \times 10^{-3} \text{M}$, $1 \times 10^{-5} \text{M}$ and $1 \times 10^{-7} \text{M}$) and lowering the [ATP] may affect the discovery of new methylation sites, due to incomplete phosphorylation of some 5' ends fragments. However, the RiboMethScores for all known rRNA positions were not affected.
12. Ethanol quantity is increased compared to the manufacturer's recommendations in order not to lose the small RNA fragments during the RNA binding to the silica membrane.
13. Do not leave the heated adapter on ice for more than 5-10 min before proceeding to the next step, this may impact your library preparation.
14. We recommend proceeding immediately with PCR amplification. However, if it is not possible, inactivate the RT by heating for 15 min at 70°C and cool down the reaction at 4°C for 1-3 hours or safely store the reactions at -20°C for overnight.
15. Make sure to use only combinations of compatible primers for barcoding. Most Illumina sequencers use a green laser (or LED) to read G and T nucleotides and a red laser (or LED) to read A and C nucleotides. Within each sequencing cycle, at least one nucleotide for each color channel must be read in the index to ensure proper reading of the barcode sequence. Use as a reference the following guide (ScriptSeqTM Index PCR primers, Illumina) for verification of barcode compatibility or check compatibility with Illumina Experimental Manager software.
16. This quantification step is crucial. Make sure to quantify all your libraries properly since an under- or overestimated quantification will interfere with subsequent sequencing reads proportion and quality.

17. The High Sensitivity DNA gel-dye mix is stable for 1 month at 4°C protected from light.

18. MINLEN parameter can vary depending on the length of the analyzed RNA, 17 nt is suitable for rRNA, but it may be shorter for tRNAs, for example.

Acknowledgement

This work was supported by joint ANR-DFG grant HTRNAMod (ANR-13-ISV8-0001/HE 3397/8-1) to MH and YM, and AO Lorraine University-Lorraine Region “Aberrant RNA methylation in cancer” funding to YM.

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Figure captions

Figure 1: General overview of the RiboMethSeq protocol.

An RNA containing 2'-O-Me residues is randomly fragmented by alkaline hydrolysis, 5'- and 3'-ends are repaired and adapters are ligated to both extremities. After amplification and barcoding, amplicons are subjected to Illumina sequencing on HiSeq1000 in single-read mode. The 2'-O-Me residues protect the 3'-adjacent phosphodiester bond from cleavage, generating a typical gap in 5'-ends coverage profile.

Figure 2: Alkaline fragmentation and RNA fragmentation control.

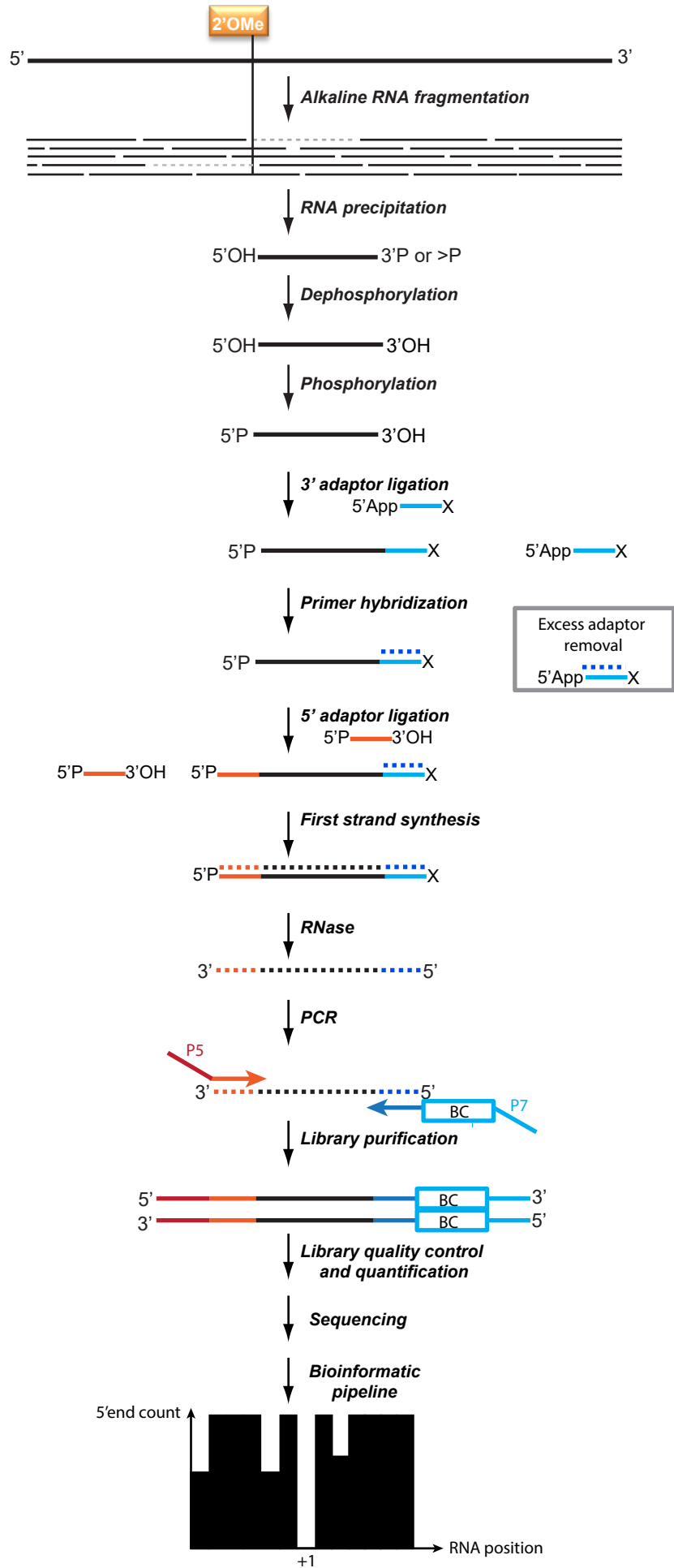
Representative capillary electrophoresis profiles of total yeast RNA (left, RNA 6000 Pico kit) and total tRNA fraction (right, Small RNA kit) fragmentation at alkaline conditions. Top -- Digital gel-like representation of the Bioanalyzer 2100 profiles with appropriate size ladder. Bottom -- Individual profiles for each sample, fragmentation times from 0 to 10 min for yeast total RNA (left) and 0 to 12 min for total tRNA fraction (right).

Figure 3: Typical profiles obtained for sequencing libraries.

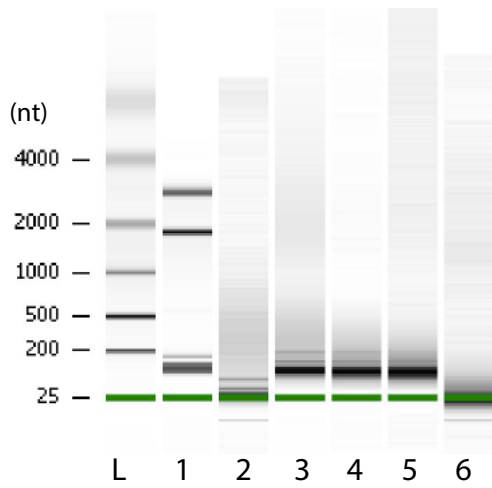
Capillary electrophoresis analysis of sequencing libraries prepared from fragmented yeast total RNA (left) and total tRNA fraction (right). Separation is done using HS DNA kit, ladder shown at the left. Libraries were prepared from total RNA fragmented for 4 min, 6 min and 10 min (left) and for total tRNA fraction fragmented for 12 min (right). Digital gel-like representations (top) and individual profiles for the corresponding libraries (bottom) are shown.

Figure 4: Bioinformatics pipeline for the RiboMethSeq analysis.

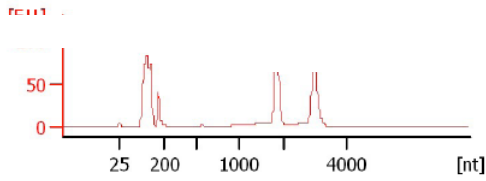
Raw reads after sequencing are trimmed using Trimmomatic to remove adapter sequences, aligned to the reference sequence by Bowtie2 and 5'-extremities of reads are counted. Calculations of ScoreMAX and RiboMethScore are performed using the spreadsheet software.



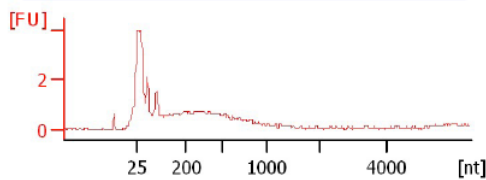
Marchand et al, Figure 1



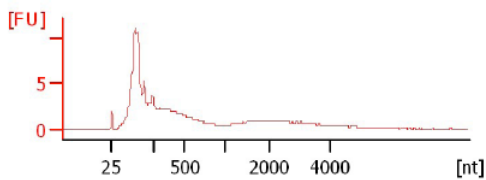
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RIN N/A



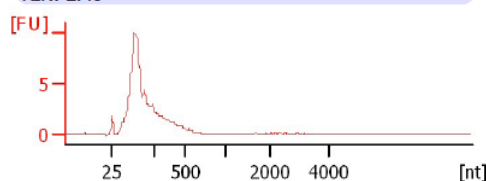
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RIN: 2.40



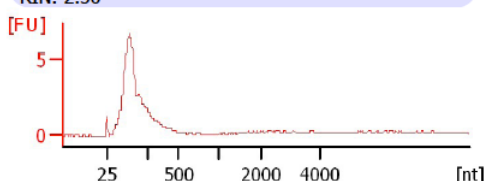
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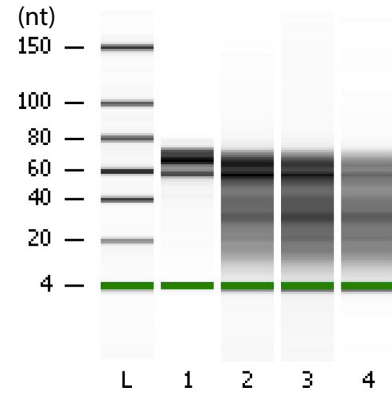
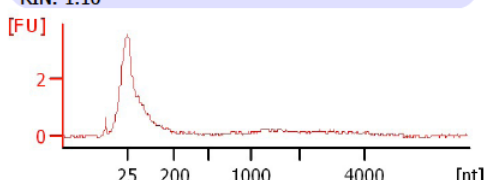
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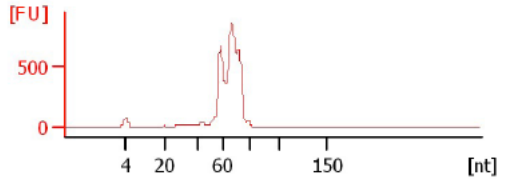
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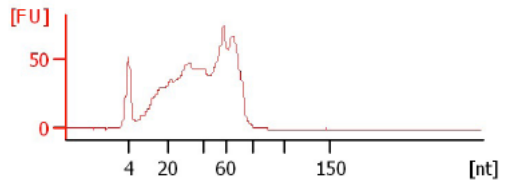
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RIN: 1.10



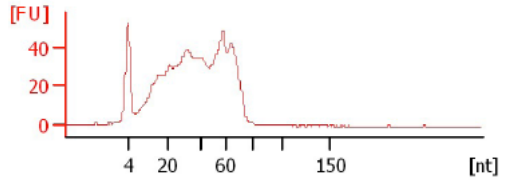
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12 % miRNA; Concentration: 808.50 pg/ul



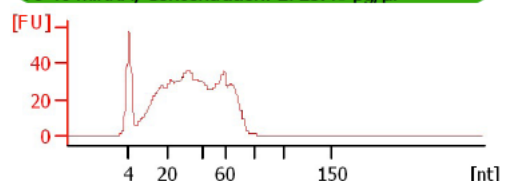
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71 % miRNA; Concentration: 3562.30 pg/ul

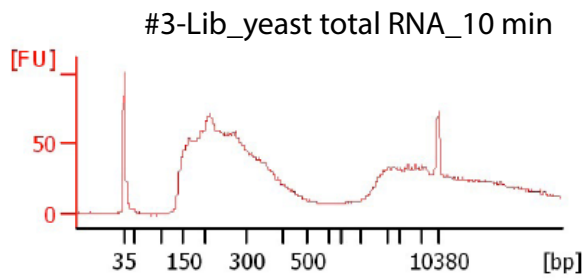
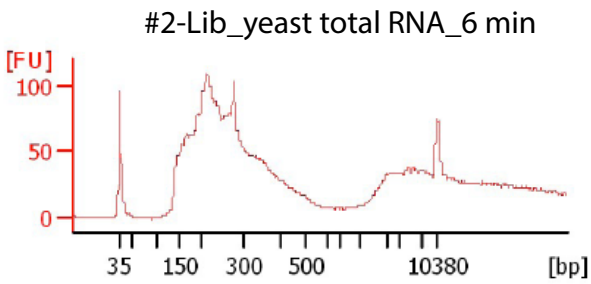
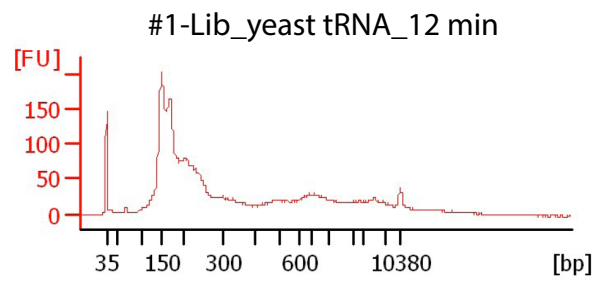
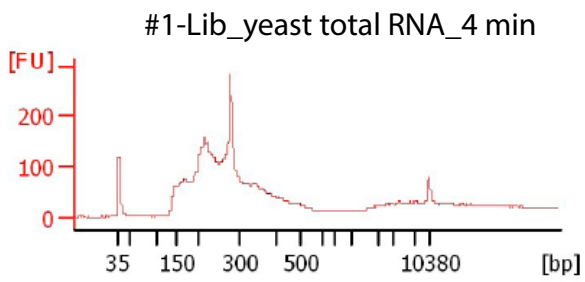
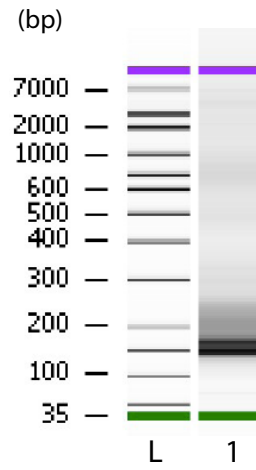
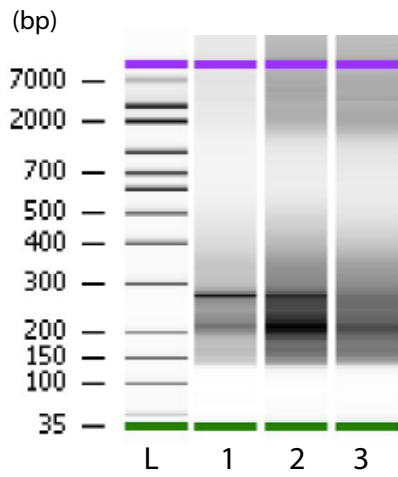


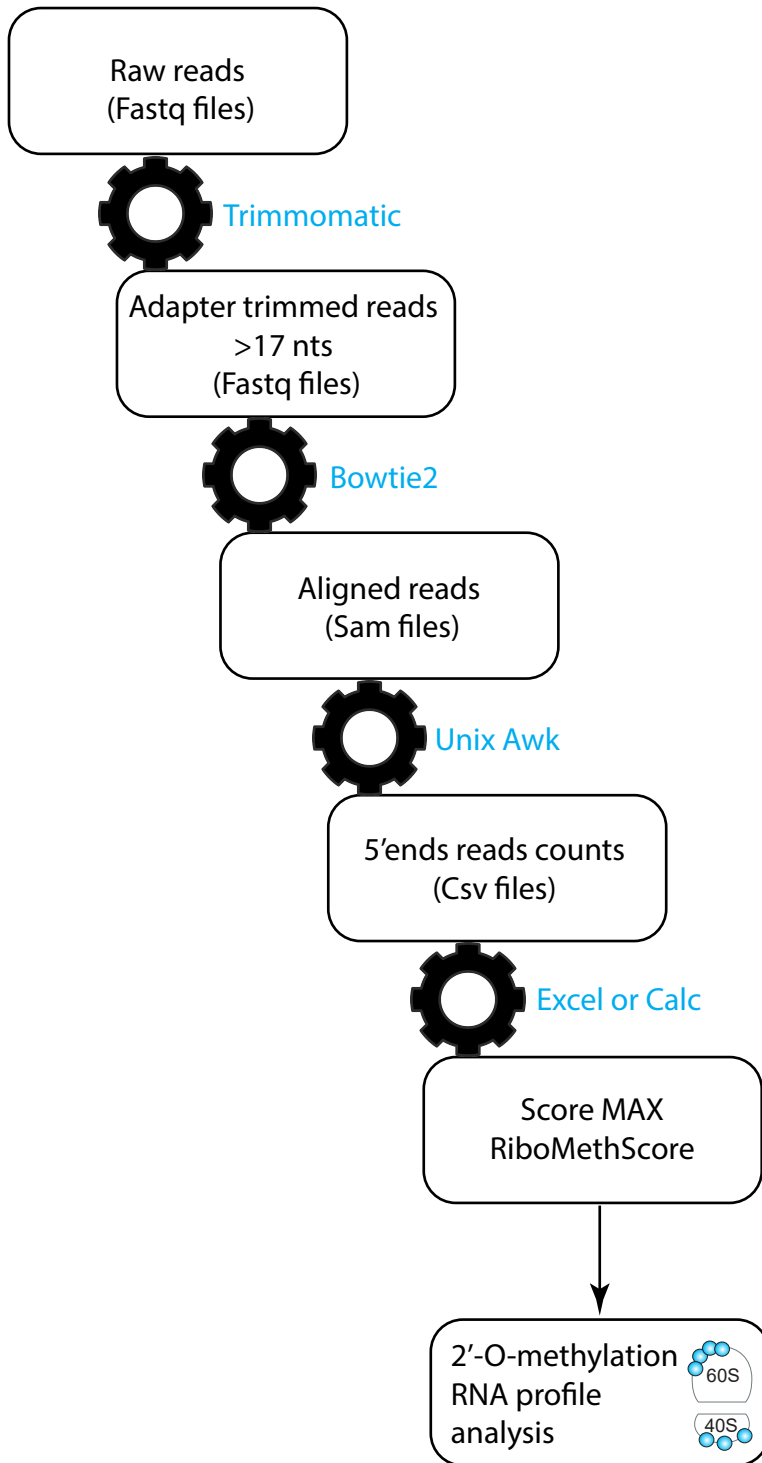
#3-yeast_tRNA_10min
75 % miRNA; Concentration: 3057.80 pg/ul



#4-yeast_tRNA_12min
79 % miRNA; Concentration: 2725.40 pg/ul







Marchand et al, Figure 4