Nm-seq

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Method Article

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Abstract

The ribose of RNA nucleotides can be 2'-O-methylated \(Nm). Despite advances in high-throughput detection, its inert chemical nature still limits sensitivity and precludes mapping in messenger RNA \(mRNA). We leveraged the differential reactivity of 2'-O-methylated and 2'-hydroxylated nucleosides to periodate oxidation to develop Nm-seq, a sensitive method for transcriptome-wide mapping of Nm with base precision. This protocol accompanies Dai et al, Nature Methods, published online May 15, 2017 \(10.1038/nmeth.4294).

Reagents

• Total RNA, DNase-treated • Molecular biology grade, RNase-free water \(Biological Industries, cat. no. 01-866-1B) • Sodium periodate \(Sigma-Aldrich, cat. No. 311448) • L-Lysine monohydrochloride \(Sigma-Aldrich, cat. no. 62929) • Ethylene glycol \(Sigma-Aldrich, cat. no. 03747) • RNA Fragmentation Reagents: Fragmentation Reagent \(10X) and Stop Solution \(Thermo Fischer Scientific, cat. no. AM8740) • RNA Clean & Concentrator \(Zymo Research, cat. no. R1015) • Shrimp Alkaline Phosphatase and CutSmart Buffer \(10X) \(New England Biolabs, cat. no. M0371S) • Antarctic Phosphatase and Reaction Buffer \(10X) \(New England Biolabs, cat. no. M0289S) • T4 Polynucleotide Kinase \(3' phosphatase minus) and Reaction Buffer \(10X) \(New England Biolabs, cat. no. M0236S) • Adenosine 5'-Triphosphate \(New England Biolabs, cat. no. P0756S) • NEBNext® Small RNA Library Prep Set for Illumina \(New England Biolabs, cat. no. E7330S) • Light-protected tubes **REAGENT SETUP** Lysine-HCl buffer, 2 M, pH 8.5: Dissolve 3.653 g of L-Lysine monohydrochloride in 10 ml of molecular biology grade, RNase-free water. Titrate to pH 8.5 with Sodium hydroxide. Sodium periodate solution, 200 mM: Dissolve 42.778 mg of sodium periodate in 1 ml of molecular biology grade, RNase-free water. Protect from light.

Equipment

Standard molecular biology lab equipment

Procedure

RNA FRAGMENTATION 1| Set up the following fragmentation reaction in a thin-walled 200 µl PCR tube. Mix well by vortex and spin down.  2| Incubate at 95 °C for 5 min in a pre-heated thermal cycler block with the heated lid closed. Remove tubes from block and immediately add 2 µl of stop solution. Mix well by vortex and spin down, and place on ice. 3| Purify fragmented RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer’s instructions. Elute in 15 µl molecular biology grade, RNase-free water. Note that each RNA Clean & Concentrator-5 column is suitable for purification of up to 10 µg RNA. **3’ END REPAIR** 4|Set up the following 3’ end repair reaction in a thin-walled 200 µl PCR tube. Mix well by gentle tapping and spin down.  5| Incubate at 37°C for 30 minutes. 6| Purify 3’ end-repaired fragmented RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer’s instructions. Elute in 15 µl molecular biology grade, RNase-free water. **OXIDATION-ELIMINATION-

DEPHOSPHORYLATION CYCLES** 7|Set up the following oxidation-elimination reaction in a dark tube. Mix well by vortex and spin down.  8| Incubate at 37 °C for 30 minutes with shaking. 9| Quench the reaction by adding 2 µl of ethylene glycol. Mix well and spin down. 10| Dephosphorylate by adding 5 µl of CutSmart Buffer \(10X), 2 µl of rSAP enzyme \(2 units) and 1 µl of molecular biology grade, RNase-free water \(total reaction volume of 50 µl), mix well and spin down. 11| Incubate at 37 °C for 30 minutes with shaking. 12| Purify RNA using RNA Clean & Concentrator-5 kit, according to the manufacturer's instructions. Elute in 32 µl molecular biology grade, RNase-free water. 13| Repeat steps 7-12 seven more times. **FINAL OXIDATION-ELIMINATION** 14| Repeat steps 7-9 once. Make sure not perform dephosphorylation \(steps 10-11). 15| Purify RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer's instructions. Elute in 32 µl molecular biology grade, RNase-free water. **5' PHOSPHORYLATION** 16|Set up the following 5' dephosphorylation reaction. Mix well by gentle tapping and spin down.  17| Incubate at 37 °C for 30 minutes. 18| Purify RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer's instructions. Elute in 8 µl molecular biology grade, RNase-free water. **LIBRARY PREPARATION FOR MASSIVELY PARALLEL SEQUENCING** 19| Use RNA from step 18 to construct a small RNA library using NEBNext® Small RNA Library Prep Set for Illumina, according to the manufacturer's instruction, with the exception that 3' adaptor ligation should be performed overnight at 16 °C.

Figures

Component	Volume (µl)	Final
Total RNA	18	10 µg
10x fragmentation reagent	2	1X
Total volume	20	

Figure 1

Table1 Table 1

Component	Volume (µl)	Final
Fragmented total RNA	43 (adjust with water)	-
Reaction Buffer (10X)	5	1X
Antarctic Phosphatase	2	10 units
Total volume	50	

Figure 2

Table 2 Table 2

Component	Volume (µl)	Final
3′ end repaired fragmented RNA	32 (adjust with water)	-
Lysine-HCl buffer, 2 M, pH 8.5	4	200 mM
Sodium periodate solution, 200 mM	4	20 mM
Total volume	40	

Figure 3

Table 3 Table 3

Component	Volume (µl)	Final
RNA from step 15	39 (adjust with water)	-
T4 PNK Reaction Buffer (10X)	5	1X
ATP, 10 mM	5	1 mM
T4 PNK (3' phosphatase minus)	1	10 units
Total volume	50	

Figure 4

Table 4 Table 4